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14. ABSTRACT

Prostate cancer is a difficult disease to treat due to its molecular heterogeneity and diverse clinical outcomes. Current therapies for treating and diagnosing prostate cancer are unsatisfactory, suggesting that new strategies and molecular markers are greatly needed. Tumor cells express specific cell surface receptor complexes for rapid growth and survival. Specific receptor-ligand complexes have profound biological functions such as cell signaling and growth. For example, androgen receptor complex plays a critical role in prostate tumor growth and response to hormone therapy. We propose to identify new receptor-ligand pairs for prostate cancer. We have developed a sophisticated targeting system to probe the tumor vasculature in vivo by phage display technology. We plan to inject phage peptides libraries into prostate tumor-bearing mice to identify specific peptides targeting to the tumor and not to the normal tissues. The tumor-specific peptides will be recovered and analyzed by molecular and biochemical methods. The tumor-specific peptides will be used as a bait to identify and clone the binding receptors by affinity chromatography and biochemical cell fractionation approaches. If we are successful, we will identify new biologically relevant receptor-ligand pairs that may be developed for therapeutic applications for prostate cancer.

15. SUBJECT TERMS
Prostate cancer, phage-display, peptide, CRKL, integrin, androgen receptor

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INTRODUCTION

Prostate cancer is a difficult disease to treat due to its molecular heterogeneity and diverse clinical outcomes. Current therapies for treating and diagnosing prostate cancer are unsatisfactory, suggesting that new strategies and molecular markers are greatly needed. Tumor cells express specific cell surface receptor complexes for rapid growth and survival. Specific receptor-ligand complexes have profound biological functions such as cell signaling and growth. For example, androgen receptor complex plays a critical role in prostate tumor growth and response to hormone therapy. It is important that more such complexes are identified for this disease. We propose to identify specific receptor-ligand pairs for prostate cancer. We have developed a sophisticated targeting system to probe the tumor vasculature in vivo by phage display technology. We plan to inject phage peptides libraries into prostate tumor-bearing mice to identify specific peptides targeting to the tumor and not to the normal tissues. The tumor-specific peptides will be recovered and analyzed by molecular and biochemical methods. The tumor-specific peptides will be used as a bait to identify and clone the binding receptors by affinity chromatography and biochemical cell fractionation approaches. If we are successful, we will identify new biologically relevant receptor-ligand pairs that may be developed into diagnostic and/or therapeutic applications for prostate cancer.

BODY

Background:

Prostate cancer is the second leading cause of cancer dearth in men and it is estimated that one in six men will develop this disease during their lifetime[1]. The cause of the disease is largely unknown. This is also compounded by the fact the disease is heterogeneous with diverse clinical outcomes. Studies have shown that tumors are heterogeneous comprising sub-population of tumor cells with different molecular properties and genetic alterations[2-4]. Some of these different properties include growth rate, metastasis, resistance to cytotoxic drugs, and cell surface receptors[3]. Moreover, the tumor microenvironment is extremely complex consisting of many cell types that can crosstalk with each other by activating and inactivating cell surface receptors[5]. Tumor cells express specific cell surface receptors that can interact with growth factors and cytokines for rapid growth, survival, and cell signaling to the extracellular environment. Specific receptor-ligand complexes can have profound biological functions. For example, in the case for prostate cancer, it is well known that androgen-androgen receptor complex plays a critical role in prostate tumor growth and response to hormone therapy[6, 7]. The complex specifically activates transcription of androgen-regulated genes and promotes cellular proliferation, survival, and differentiation[8, 9]. However, there are a limited number of receptor-ligand pairs that have been thus far identified for prostate cancer. We propose to identify specific receptor-ligand pairs for prostate cancer by in vivo phage display. This approach has not been explored for targeting prostate tumor in vivo. Identifying the molecular receptor-ligand complexes during tumor development is an important step towards developing new diagnostic markers and molecular therapeutic targets for prostate cancer.

Statement of work:

Task 1. To isolate and characterize peptides targeting prostate tumor cells in vivo by phage peptide libraries (1-18 months).

- We will generate 30 tumor-bearing mice (human prostate cancer xenographs) for the in vivo screening (1-2 months). **Completed.**
- We will inject phage peptide libraries into the tumor-bearing mice and isolate tumor-specific homing phage peptides after three or four rounds of in vivo selection. We will use several different peptide libraries (3-7 months). **Completed.**
- We will characterize the binding and inhibition properties of the tumor homing peptides in vitro and in vivo. We will generate 30 tumor-bearing for the in vivo studies. Prostate cancer cell lines including DU145, PC-3, and LNCaP will be used for the in vitro studies (8-12 months). **Completed.**
- We will characterize the tissue localization of the tumor-specific homing phage peptides by immunohistochemistry. Tissue samples from the tumor bearing mice and prostate cell lines will be used for these studies (13-18 months). **Completed.**

Task 2. To identify and validate molecular receptors binding to the tumor homing peptides (18-36 months).

- We will analyze tumor-specific peptides by searching the protein database for potential biologically relevant receptor leads (18-20 months). **Completed.**
- We will use biochemical cell fractionation and affinity chromatography to purify the receptor binding to the tumor-specific peptides. We will select one or two most promising peptides for receptor identification (21-30 months). **Completed.**
- We will clone the receptors and characterize them in the context of the tumor homing peptides (31-36 months). **Completed.**

Results reported from the second annual report:

We have shown that CRKL is important in prostate cancer and interacts with β_1 integrin on the cell surface, we next investigated the downstream signalling mechanisms of CRKL activation and if this might have any direct functional consequences with the androgen receptor. To our surprise we found that in addition to the β_1 integrin, we found that CRKL and AR are in a complex (Fig. 1). This is an important find because it demonstrates for the first time that an intracellular signalling adaptor molecule such as CRKL could interact with AR. This is in contrast to the already known partners of the AR, which are nuclear proteins[15,16].

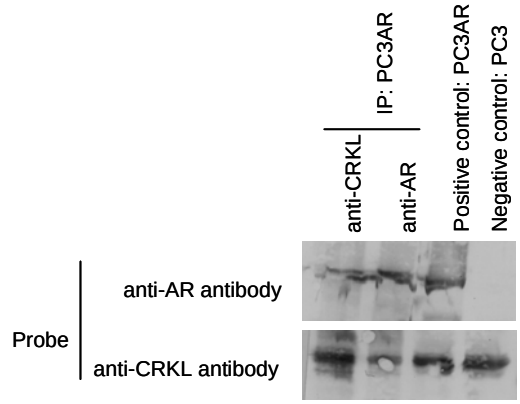


Figure 1. AR and CRKL complex. Co-immunoprecipitation from PC3AR cells.

To understand the functional role of the AR-CRKL complex, a CRKL expression construct was investigated in an AR transcriptional reporter assay. We first co-transfected CRKL and an AR reporter into COS cells and measured its activity. We found that CRKL could transcriptionally activate the AR when stimulated with the hormone mibolerone (Fig. 1a). This result prompted us to further examine this event in prostate cancer cells. A similar transactivation was observed in androgen-independent PC3 cells (Fig. 1b) and interestingly, also found that CRKL was phosphorylated (Fig. 1b, inset) suggesting that phosphorylated CRKL is recruited to the AR complex and acts as a co-activator.

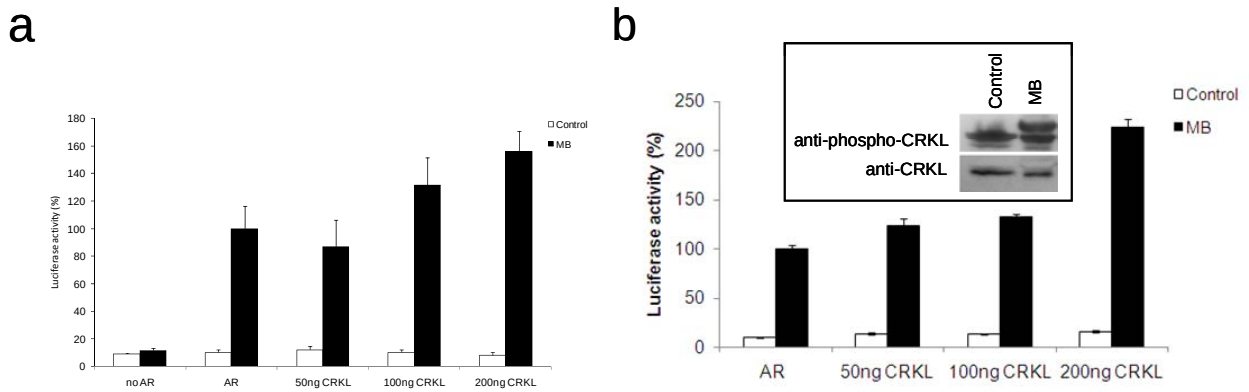


Figure 2. Functional reporter assay. AR and CRKl were co-transfected into PC3 prostate cancer cells and its transcriptional activity was measured. Cell lysates were used to probe for phosphorylated CRKL.

Encouraged by these initial results and since CRKL is distributed both in the cytoplasm and nucleus[10,11], we next wanted to determine the sub-cellular distribution of AR-CRKL complex in prostate cancer cells. A significant co-localization between CRKL and AR was observed in PC3 cells when co-transfected with the AR and CRKL constructs or in PC3AR cells when transfected with the CRKL construct (Fig. 3a,b). A similar staining pattern was also observed in the LNCaP cells (Fig. 3c). When stimulated with the hormone mibolerone, CRKL localised mostly to the nucleus, whereas in the presence of the AR inhibitor casodex, a more diffuse distribution was observed. It is possible that the AR and CRKL may form a complex in the cytoplasm when stimulated by the steroid and translocate into the cell nucleus. Since CRKL has a nuclear export signal[12] it has the capability of exiting back into the cytoplasm where it can interact with the AR. Taken together, these data provide evidence of co-localisation of AR and CRKL in androgen-independent prostate cancer cells.

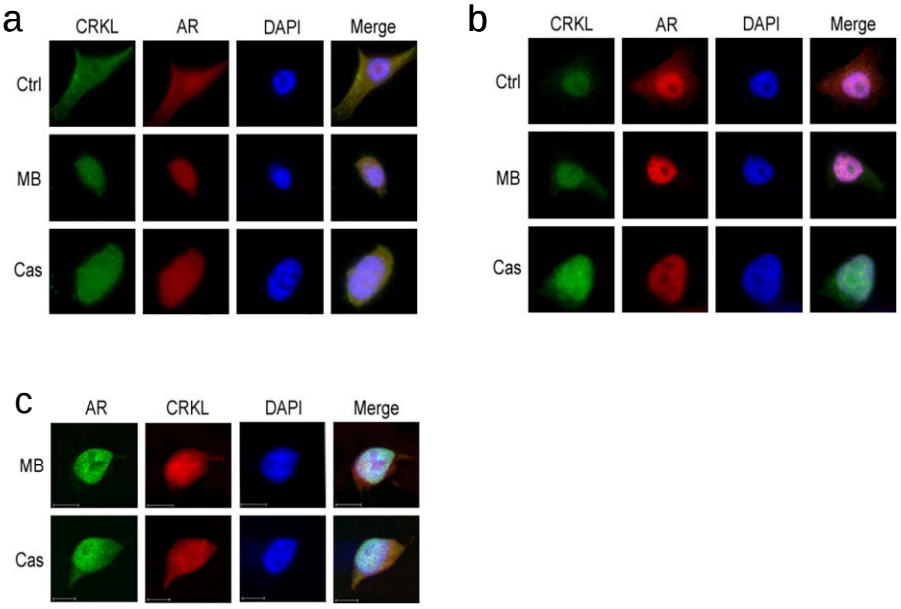


Figure 3. Co-localisation of AR and CRKL complex. PC3 cells were co-transfected with AR and CRKL (**a**), PC3AR cells transfected with CRKL (**b**), and LNCaP cells transfected with CRKL (**c**). The transfected cells were stimulated with milbolerone or inhibited by casodex.

In summary, we have identified a couple of new receptor-ligand complexes in prostate cancer cells. The association between β_1 integrin/CRKL and AR/CRKL are novel discoveries and this is the first report of such findings. We have published the work characterising the association between β_1 integrin/CRKL (Mintz et al., 2009, please see the appendix). We have partially charaterised the novel interaction between the AR and CRKL as stated in the second annual report. We plan to continue and complete the studies.

Continuation from the second annual report to the final reporting period:

In this report, we have completed (Task 2) the characterisation of the novel interaction between AR and CRKL. Specific details are described below. In addition, the culmination of experiments completed for Task 2 has been prepared and submitted for a publication to a peer-review journal (please see appendix, Reebye et al., 2009).

In the second annual report we found that in addition to the β_1 integrin, CRKL and the AR are in a complex in prostate cancer cells. In addition to the stable cell line expressing the AR in PC3 cell line as previously reported, we examined a prostate cell line endogenously expressing the AR (LNCaP) that is used for the prostate cancer research. Similar results were found by co-reciprocal immunoprecipitation experiments showing that CRKL and the AR are in a complex (Fig.1a). We also found that CRKL redistribute to the nucleus when stimulated with the androgen hormone (Fig.1b).

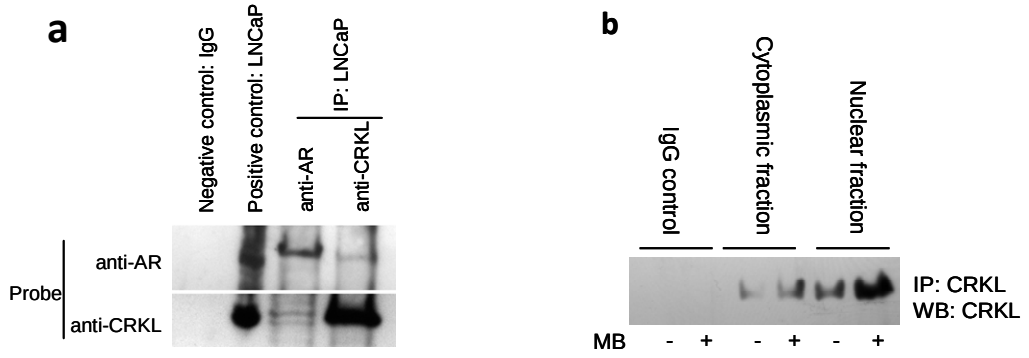


Figure 1. AR and CRKL complex. (a) Co-immunoprecipitation of AR and CRKL from LNCaP whole cell extract. (b) Immunoprecipitation of CRKL from the different cellular compartments (cytoplasmic and nuclear fractions).

Since we have demonstrated that CRKL is predominantly re-distributed into the nucleus and co-localises with the AR when over-expressed, we next addressed whether the AR-CRKL interaction was a bona-fide complex at the transcriptional level by performing a chromatin immunoprecipitation (ChIP) assay in LNCaP cells treated either in the absence or presence of hormone to confirm that endogenous CRKL and the AR were both present on the enhancer region of the androgen regulated human Kallikrein 2 (KLK2) gene (Fig. 2a). KLK2 is widely known as prostate specific antigen (PSA) as they both share high homology (13). KLK2 also adds significant information when detecting prostate cancer (14-18). To understand the functional role of the AR-CRKL complex, a

CRKL expression construct (pCDNA-CRKL) was then used in an AR dependent transcription luciferase reporter assay in COS cells and PC3 cells. We found that overexpression of CRKL enhanced hormone induced activity of the AR (Fig. 2b and c). To substantiate the reporter assays, we then measured the effects of CRKL overexpression on endogenous PSA levels in LNCaP cells treated with mibolerone or the AR inhibitor, casodex. There was a significant upregulation of PSA when CRKL was overexpressed relative to the control (empty vector transfection) (Fig. 3). We also observed this effect in the absence of hormone stimulation and in cells treated with casodex suggesting that CRKL may act as a strong coactivator in LNCaP cells.

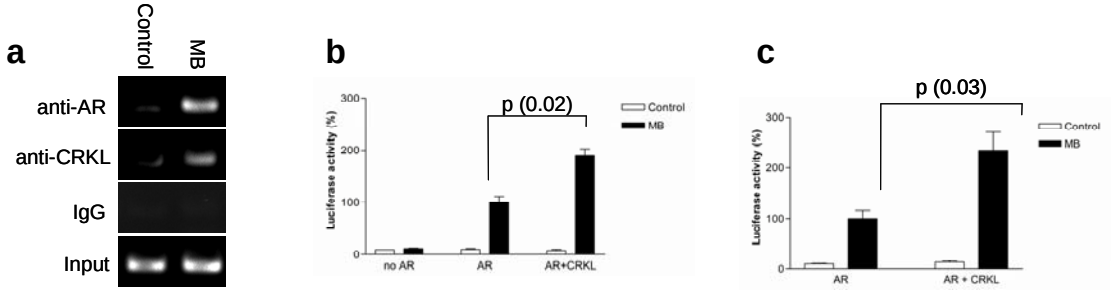


Figure 2. A. Chromatin immunoprecipitation on LNCaP cell extract with anti-AR and anti-CRKL antibodies at an endogenous androgen-responsive element. B and C. AR transcriptional activity in COS.and LNCaP cells transfected with CRKL. Results are the mean ± SEM (from 4 independent duplicate experiments) and statistically significant, $p < 0.05$ (Two tailed Student's t test).

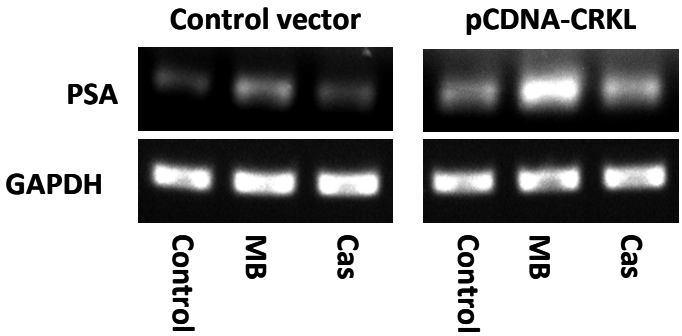
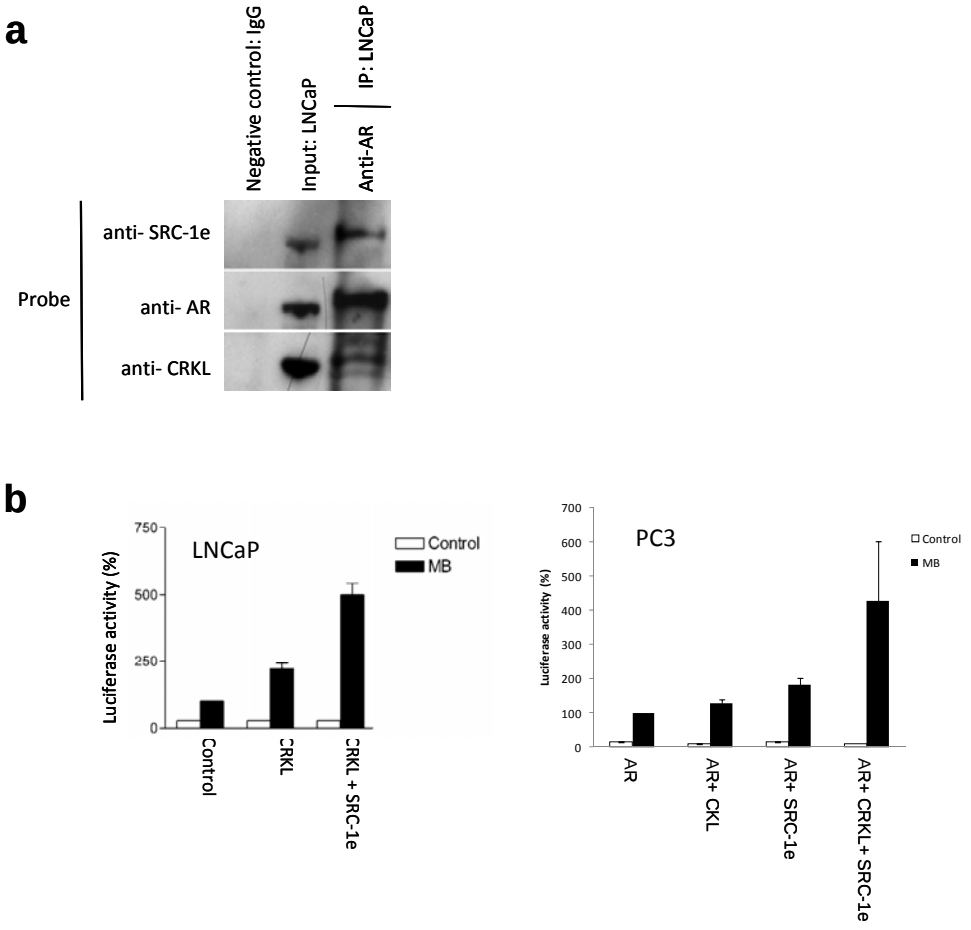


Figure 3. Functional PSA level in LNCaP cells transfected with CRKL. Total RNA from LNCaP cells overexpressing CRKL and treated with mibolerone (MB) or casodex (cas) were reverse transcribed for PCR amplification of PSA and GAPDH for semi quantitative analysis. Results are representative of three individual experiments.

These studies suggest that CRKL and the AR are in a transcriptionally functional complex. Moreover, CRKL may act as a strong co-activator in expressing PSA level in prostate cancer cells.

Since the AR forms a large complex in the nucleus, we next determined whether CRKL also interacts with any known AR co-activators. We chose the most characterised activator of the steroid receptor superfamily, SRC-1e (a histone acetyltransferase and member of the p160 family of steroid receptor co-activator) (19). We found that CRKL and SRC-1e are in a complex with the AR when CRKL and SRC-1e were simultaneously overexpressed in LNCaP cells (Fig. 4a), and that AR transcriptional

activity was significantly enhanced (Fig. 4b). To gain greater insight into the transcriptional activity of the AR-CRKL complex, we tested what effect CRKL overexpression would have on an AR mutant (ARΔLBD) missing its ligand binding domain. This mutant AR contains only the activation function 1 (AF1) and the DNA binding domain (DBD) of the AR (20) (Fig. 4c). Since AF1 is the predominant transcriptional activation domain of the AR (21-23), this mutant remains constitutively active even in the absence of hormone stimulation (21). When ARΔLBD along with CRKL were co-transfected into AR negative PC3 prostate cells, an enhanced transactivation was observed (Fig. 4c). This was similar to that seen with SRC-1e (also known to interact at the AF1 domain of the AR) (22). Since overexpression of both CRKL and SRC-1e further enhanced activity of ARΔLBD, it may be possible that there is a synergistic effect of both proteins at that region of the AR. The AF1- region also contains several potential phosphorylation sites (24) and interaction domains for co-activators (25-30), therefore, the influence of CRKL could be mediated via two important properties of its SH domains. First, they contain important protein-protein interaction modules; and secondly they are non-catalytic regulators of kinase activity (31). We next assessed if the truncated SH domains of CRKL would still have an effect on the constitutively active ARΔLBD mutant by performing transcriptional reporter assays with the SH2, the N-terminal SH3 (SH3-N) and the C-terminal SH3 (SH3-C) domains of CRKL. We found that all three SH domains were responsible in mediating transcriptional activity of the AR (Fig. 4d). CRKL may therefore potentially assist in the assembly of a heterogeneous multi-protein complex on the AR, involving key kinase signalling factors that have yet to be identified.



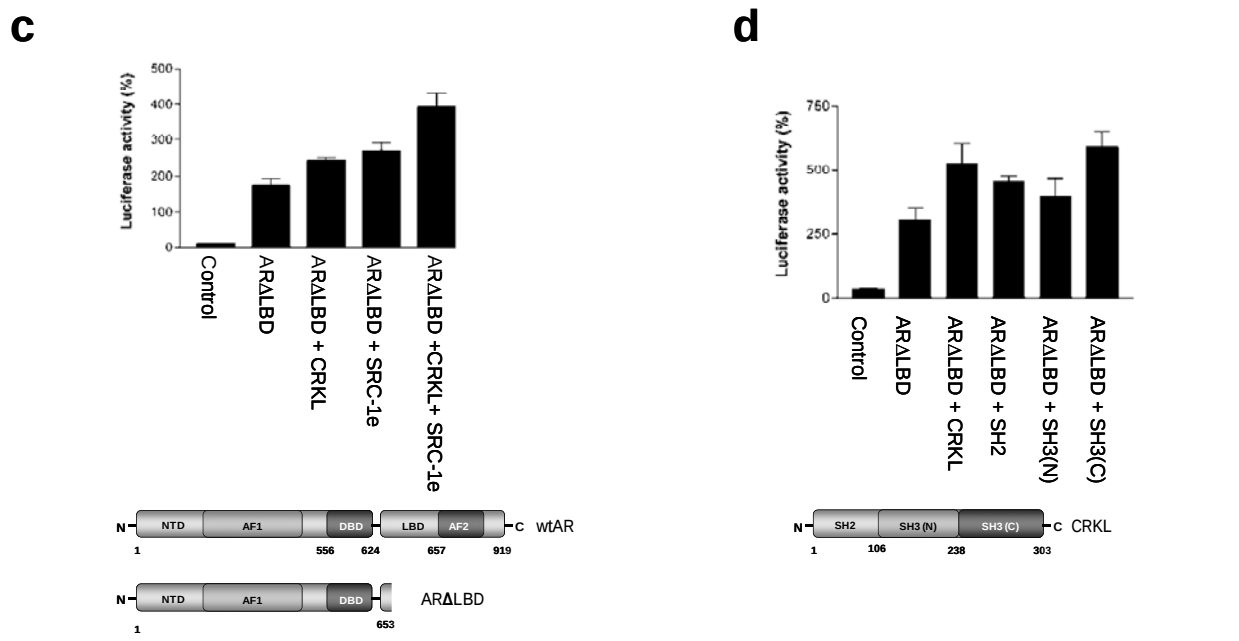


Figure 4. Transactivation of CRKL, AR, and SRC-1e complex. A. Immunoprecipitation of CRKL, AR, and SRC-1e complex. LNCaP cell lysates were immunoprecipitated with anti-AR antibody or with IgG as control and probed with anti-AR, anti-CRKL, and anti-SRC-1e antibodies for a western blot analysis. Representative data is shown. (The slight band shift seen in the IP lane is attributed to the high salt buffer used for the sepharose bead washing conditions following immunoprecipitation). B. Androgen stimulated AR luciferase reporter assay where AR, CRKL, and SRC-1e are co-transfected into LNCaP and PC3 cells. C. Enhanced transactivation of the ARΔLBD mutant following overexpression of CRKL alone or CRKL with SRC-1e, or D., following overexpresion of the CRKL fragments: SH2, SH3(N) and SH3(C). The ARΔLBD mutant is a constitutively active and nuclear AR mutant with the LBD and AF2 domains (36). Results are the mean ± SEM from 3 independent duplicate experiments. The schematic diagrams show the ARΔLBD mutant and the location of CRKL SH domains.

Studies in the hormone refractory stage of prostate cancer have shown that the AR still remains functionally active in the absence of androgens. Amplification and mutations of the AR (32) in addition to growth factors may also modulate AR activity under such conditions suggesting a cross-talk between these factors and the AR signalling pathway (33). Therefore, we next asked whether the CRKL/AR/SRC-1 complex can be activated by an alternative downstream signalling pathway involving growth factors. In order to determine whether AR transcriptional activity of the complex is directly dependent on CRKL, PC3 cells were transfected with or without CRKL followed by co-stimulation with growth factors and casodex. We found a significant level of AR transcriptional activity only in cells where CRKL was over expressed (Fig. 5) suggesting that CRKL is a critical component of the AR complex to overcome the inhibitory effects of casodex. It is possible therefore that the activation of an alternative signalling pathway by growth factors may be recruiting CRKL to associate with the AR complex and act as a co-activator.

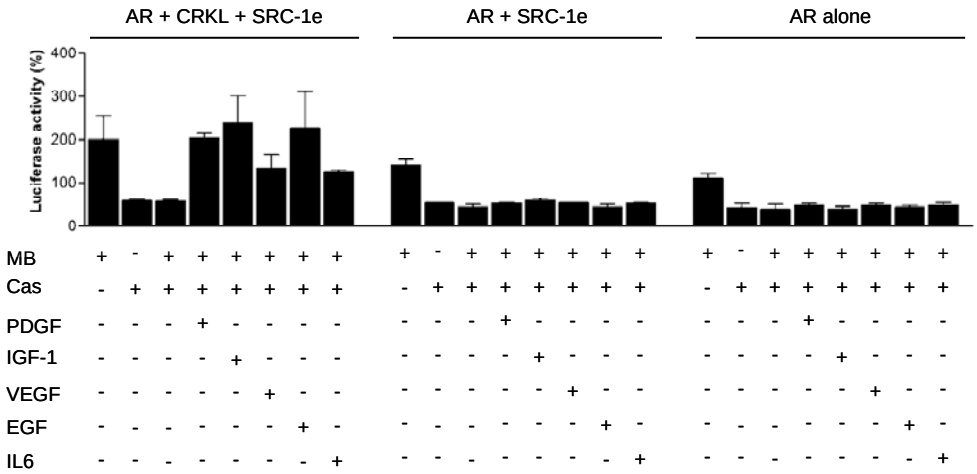


Figure 5. AR driven luciferase reporter assay in PC3 cells transiently transfected either with (AR, CRKL and SRC-1) or (AR and SRC1). Growth factor and cytokine (IL6) induced AR activity was measured in the presence of casodex and low level mibolerone. Increased AR activity was observed only in cells where CRKL was overexpressed. Results are the mean ± SEM from 3 independent duplicate experiments.

Since we demonstrated that growth factors are able to activate the CRKL/AR/SRC-1 complex independent of hormone stimulation (Fig.5), we next sought a possible downstream signalling mechanism. As some of the growth factors that we have used are known to be associated with the ERK/MAPK signalling pathway; we used a specific inhibitor of the ERK/MAPK pathway-U0126 for subsequent reporter assays. We found that U0126 abrogated phosphorylation of ERK (Fig. 6a) and significantly reduced EGF induced AR transcriptional activity (Fig. 6b). Although this suggests that the ERK/MAPK pathway may be involved, several other growth factors could also activate the ERK/MAPK pathway. We therefore targeted the EGFR axis by using a specific inhibitor of this receptor (AG1478) which abrogated phosphorylated ERK (Fig. 6a). We found that targeting the EGFR pathway significantly reduced AR transcriptional activity (Fig. 6b) suggesting that EGFR is one of the many mechanisms involved in activating androgen independent AR transcription. Although EGFR induced AR signalling has already been established (34,35), we provide data to show that important co-regulators (such a CRKL) can be a sufficient driving force to assist the AR in adopting this alternative signalling pathway.

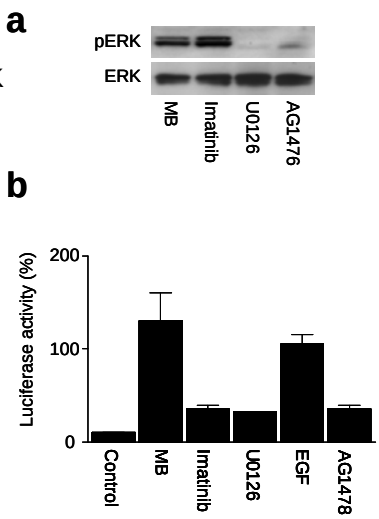


Figure 6. The CRKL/AR/SRC-1 complex associated with the ERK/MAPK signalling. (**a**) Western blot analysis of cells after treatment with the androgen mibolerone (MB), imatinib (Inhibitor of phosphorylated CRKL), UO126 (ERK/MAPK inhibitor) and AG1478 (EGFR inhibitor) to show its effect on the phosphorylation status of ERK and CRKL. (**b**) AR driven luciferase reporter assay in PC3 cells transiently expressing CRKL/AR/SRC-1 complex and treated with mibolerone (MB) or EGF in the presence of imatinib, UO126 or AG1478. Results are the mean ± SEM from 3 independent duplicate experiments.

To gain further insight into the biological and cellular influence of CRKL on AR signalling we performed siRNA knockdown studies in LNCaP cells. After establishing efficient knock down of CRKL, SRC1 and AR (Fig. 7a), we used the same siRNA conditions to carry out a reporter assay in response to androgen stimulation (Fig. 7b). Androgen induced AR activity was significantly reduced when CRKL, AR and SRC-1 were targeted by siRNA. Since we had already established that endogenously expressed CRKL was recruited with the AR to the androgen regulated PSA gene in LNCaP cells (Fig. 3), we next established whether knockdown of CRKL or overexpression of CRKL would have an effect on recruitment of AR to the same enhancer region of PSA. A ChIP assay was performed in siRNA-CRKL transfected cells and in cells with transient overexpression of CRKL (pCDNA-CRKL) (Fig. 6c). The amount of ligand activated AR recruited to the enhancer region of the endogenous androgen regulated PSA gene was significantly different in cells with overexpression of CRKL compared to cells where CRKL expression was knocked down (Fig. 7c).

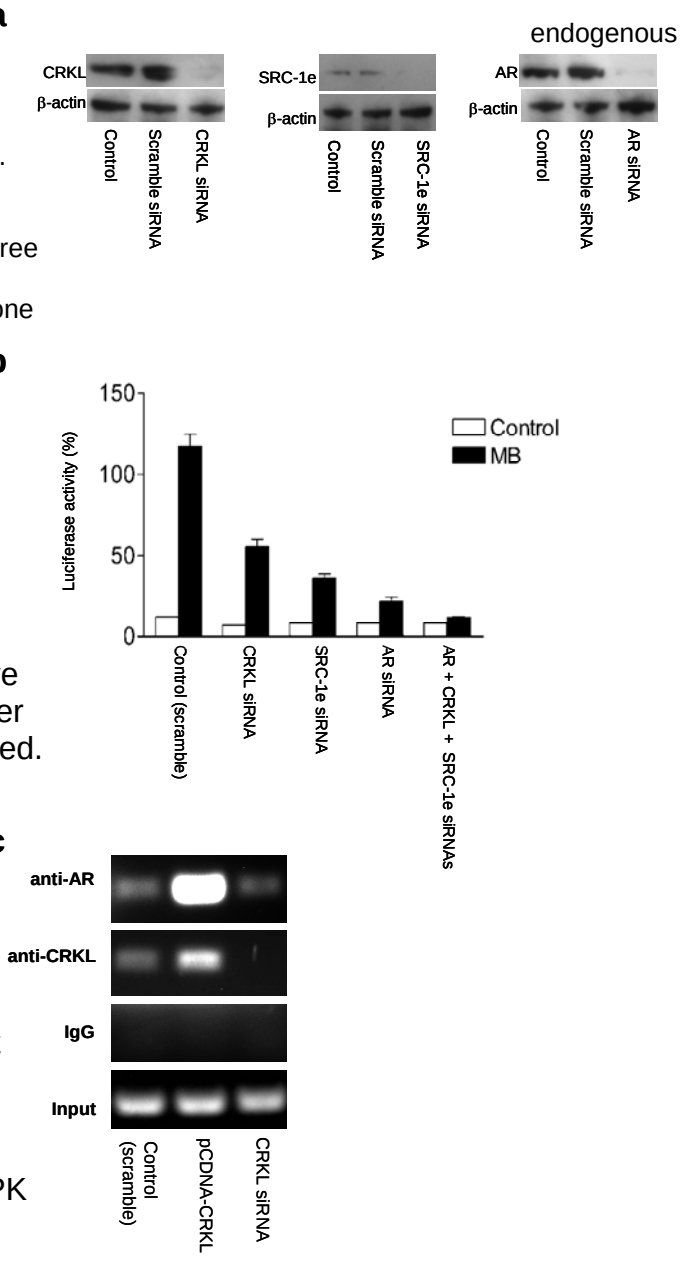


Figure 7. siRNA knockdowns of AR, CRKL and SRC-1 affects AR activity. (**a**) Western blot for targeted knockdown of CRKL, SRC-1 and AR in LNCaP cells 72 hours post transfection. β-actin is used as a loading control. (**b**) AR driven luciferase reporter assay of ligand activated AR following targeted knock down of either CRKL, AR, SRC-1 or knock down of all three (CRKL/ AR/SRC-1) in LNCaP cells. (**c**) ChIP analysis of the PSA enhancer region in mibolerone (MB) treated LNCaP cells following targeted knockdown of CRKL by siRNA or transient over-expression of CRKL (pCDNA-CRKL). Representative data is shown. Results for the reporter assay are the mean ± SEM from 3 independent duplicate experiments.

In conclusion, we show that the adaptor protein, CRKL, has a dynamic function where in prostate cancer it influences AR signaling via a cooperative interaction with the AR and possibly other co-activators that are yet to be determined. More importantly this interaction appears to be modulated by growth factors in a hormone free environment even in the presence of casodex. There are as yet no published reports that define the role of an adaptor molecule in hormone resistant prostate cancer cells. The next challenge will be to map the interaction domain of CRKL with the AR, and to establish which other CRKL associated factors are involved along the ERK/MAPK signal transduction pathway to facilitate androgen independent signaling.
The possible existence of other coactivators similar to CRKL will have broader

implications when further defining the alternative pathways by which the AR adapts in different therapeutic settings.

In summary:

This final report contains all the experimental data for the duration of the grant period. The results and the completion of Task 1 was reported in the first annual report with the successful publication in a peer-review journal *PNAS* (Mintz et al., 2009, please see appendix). In addition, a review article regarding the work published in *PNAS* has been accepted in a peer-review journal, *Cell Cycle* (please see appendix, Ozawa et al., 2010). For Task 2, we have successfully performed all the necessary experiments to functionally characterize and validate the new molecular complex—CRKL/AR in prostate cancer. The culminating results from Task 2 has been prepared and submitted for a publication in a peer-review journal for 2009 (Reebye et al., 2009, please see appendix). We are very grateful for the funding from the Department of Defense (DOD). This work could not have been possible without the funding from DOD.

MATERIALS AND METHODS

Cell Culture

PC3 and LNCaP cells (American Type Culture Collection) were cultured in RPMI-1640 (Sigma) supplemented with 100 units/ml penicillin, 0.1mg/ml streptomycin, 2mmol/L glutamine (Sigma) and 10% fetal bovine serum (Labtech International). PC3wtAR cells (28) were grown in RPMI with 4µg/ml of Geneticin (Gibco). Androgen-free culture conditions were carried out in phenol red free DMEM or RPMI supplemented with charcoal-stripped fetal bovine serum (Labtech International).

Reporter assay

The following plasmids have been described previously: pSG5-SRC-1e (36)**,** pSVAR (ΔLBD) (a.a. 1-653) (20) and pCDNA3.1-CRKL (37). The following were kind gifts to Bevan CL: pSVAR from Brinkmann A (Rotterdam), TAT-GRE-EIB-Luc from Jenster G (Rotterdam). Cells were cultured in 24-well plates for 24 hours followed by transfection using FuGENE6 (Roche Diagnostics), or for LNCaP cells by using Nanofectamin (PAA). The transfected DNA (measured in nanograms per well) included empty pSG5 or empty pCDNA3.1 control plasmids to standardise the amounts of DNA, the reporter TAT-GRE-E1B-Luc (500ng), pdmLacZ-β-Gal (250ng) and the vectors pSVAR (50ng), pSVAR- ΔLBD (50ng), pSG5-SRC-1e (200ng) and pCDNA3.1-CRKL (50-200ng). After incubation for 16 hours, cells were washed and treated with 10nM hormone mibolerone (MB) for 24hours. Cells were washed twice in phosphate buffered saline (PBS) and lysed in reporter lysis buffer (Promega). Extracts were analysed for firefly luciferase activity using the LucLite™ kit (Packard) and values corrected for β-galactosidase activity measured by the GalactoLight Chemiluminescence assay (Tropix).

Inhibition studies
PC3 cells were transfected as described above for 24 hours. For single treatment with growth factors alone: PDGF (20ng), IGF-1 (20ng), VEGF (100ng), EGF (100ng) and IL6 (100ng) were added to the cells for 16 hours prior to harvesting for the reporter assay. Where cells were pre-treated with either MB (10nM) or casodex (Cas) (1μM): compounds were added for 4 hours and washed twice in TBS before adding the growth factors: PDGF (40ng), IGF-1 (40ng), VEGF (200ng), EGF (200ng) and IL6 (200ng) for 4 hours prior to harvesting. Where cells were given a combination of treatment for 16 hours (Cas + MB + growth factors + U0126): 1μM of Cas alone or 1nM of MB and 10μM of Cas combined were added to the cells together with the growth factors at the same concentrations used for the 16 hour incubation period as sated above; and the MAPK/ERK kinase inhibitor U0126 (20μM) prior to harvesting. Where cells were treated with MB (10nM) or the growth factors, and imatinib (5μM), U0126 (20μM) or AG1478(20μM): cells were pre-treated with the growth factors at the same concentrations used for the 4 hour incubation period, as stated above. Cells were washed twice in TBS prior to addition of imatinib, U0126 or AG1478 for a further 4 hours prior to harvesting.

Immunoprecipitation
Immunoprecipitation (IP) was performed as previously described (23) with some modifications. Cells were grown to 80% confluency in a 10 cm culture dish**.** 10nM MB was added for 2 hours before harvesting. Cells were washed twice in ice-cold PBS before incubation for 20 minutes on ice in IP buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% Nonidet P-40, 1mM dithiothreitol and complete protease inhibitor cocktail). The lysates were centrifuged at 14000 rpm for 5 minutes at 4°C. 500μg of total protein extract was then pre-cleared with protein-A/G-Ultralink Resin (Thermo Scientific) for 45 minutes at 4°C prior to incubation with rabbit primary antibody against AR or CRKL (Santa Cruz) overnight at 4°C. The immune complex was then precipitated with protein-A/G-Ultralink resin for 1 hour at 4°C, washed three times in a high salt wash buffer (20mM Tris pH 8, 150mM NaCl, 1mM EDTA, 0.1% NP40) and resuspended in laemmli SDS loading buffer for separation by SDS-PAGE.

Immunoprecipitating CRKL from Cytoplasmic and nuclear cell extracts
Cells (at a density of $0.7x10^6$ cells/ml) were grown for 16 hours in $10cm^2$ dish and transfected with pCDNA3.1-CRKL or empty vector control for 24 hours followed by hormone treatment for a further 2 hours. Cells were washed twice in cold TBS and then gently scraped into an eppendorf. Cells were then resuspended in 100μl of cold homogenisation buffer (10mM Hepes, pH7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT and 0.5mM PMSF). Harvested cells were allowed to swell on ice for 15 minutes before being lysed by the addition of 10μl of a 10% solution of NP-40 followed by 10seconds of vigorous vortexing. The resulting nuclear pellet was then centrifuged at 13,000rpm for 30 seconds with the cytoplasmic supernatant removed. The nuclear pellet was then washed three times in homogenisation buffer containing NP40 and resuspended in 100μl of the same buffer. The nuclear proteins were solubilised by sonication. 100μl of the cytoplasmic and nuclear extracts were resuspended in 400 μl of IP buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% Nonidet P-40, 1mM dithiothreitol and complete protease inhibitor cocktail). After a pre-clearing stage with protein-A/G-Ultralink Resin (Thermo Scientific) for 45 minutes, 1μg of anti-CRKL (Santa Cruz) or IgG as negative control was added to each sample and incubated overnight at 4˚C. The

immune complex was then precipitated with protein-A/G-Ultralink Resin for 1 hour at 4˚C, washed three times in PBS and resuspended in laemmli SDS loading buffer for separation by SDS-PAGE.

Immunofluorescence
PC3wtAR cells were grown to 50% confluency in normal RMPI media on sterile glass coverslips in 24-well plates. For transient overexpression of CRKL, 200ng of pCDNA3.1-CRKL was transfected using FuGENE6 (Roche) according to the manufacturer's instructions. Cells were then grown for an additional 24 hours. The coverslips were then washed in PBS three times, fixed in 4% paraformaldehyde for 20 minutes and solubilised in 0.2% TritonX100 for 20 minutes. The coverslips were washed three times followed by treating with 10% foetal calf serum for 45 minutes. Rabbit anti-AR (1:200) (Santa Cruz); and Mouse anti-CRKL (1:50) (Cell Signalling Technology) were added to the cells in 10% FCS for one hour. Cells were washed three times in PBS and 10% FCS added for 15 minutes before incubation with Alexa-488 conjugated goat anti-mouse (1:600) or Alexa-594 conjugated chicken anti-rabbit secondary antibody(1:600) (Molecular Probes) for one hour. After five washes in PBS, coverslips were mounted on glass slides with Vectashield containing 4'6'-diamidino-2-phenylindole (DAPI) (Vector labs). Slides were visualised on a Leica DM4000 at 100x magnification. An average of 10 images per treatment was captured.

Immunohistochemistry
Prostate samples from 6 separate patients were obtained from AccuMAx Array (Cepheid). The formalin fixed, paraffin embedded prostate tissue were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Endogenous peroxidise activity was blocked using 2% hydrogen peroxide. Antigen retrieval was carried out by microwaving at 750W in 0.01M trisodium citrate, pH6, for 5 minutes. Sections were blocked with PowerBlock (BioGenex) (1:10 dilution) for 3 hours at room temperature and incubated with the primary antibody: anti-human CRKL (Santa Cruz) (1:250) overnight at 4˚C. After several washing, sections were incubated with biotinylated anti-rabbit IgG (DAKO) (1:200 dilution) for 45 minutes, followed by peroxidise conjugated with streptavidin (DAKO) (1:100 dilution) for 30 minutes. Sections were then washed and enzyme activity was developed in 1mg of 3,3'diaminobenzidine tetrahydrochloride (DAKO) per ml and counterstained with hematoxylin (Vector Laboratories). All images were processed at x40 magnification with an Olympus CKX41 microscope mounted with a CC12 Olympus UCMAD3 camera.

Chromatin immunoprecipitation (ChIP)
LNCaP cells were seeded at a ratio of $7x10^5$ cells/ml in serum starved media for 24 hours in 6 well plates followed by transient overexpression using Lipofectamine RNAiMAX (Invitrogen) with (200ng pCDNA3.1-CRKL), siRNA knockdown of CRKL (Dharmacon) as previously mentioned or un-transfected for 48 hours. After a total of 72 hours, cells were treated with 100nM MB and 200ng EGF for 2 hours before cross-linking with formaldehyde (Sigma) for 10 minutes at 37˚C. ChIP was performed using the Upstate Chromatin Immunoprecipitation kit (Upstate) following the manufacturer's instructions. Briefly, cross-linked cells were lysed and sonicated for chromatin fragments of about 1000bp. Anti-AR (Santa Cruz), anti-CRKL (Cell signalling) or Rabbit-IgG (Jackson Laboratories) were incubated with the fragments to precipitate out their corresponding peptides cross-linked with the DNA fragments. The cross-linked DNA

was then recovered by protease treatment and phenol-chloroform extraction and semi-quantitative PCR performed using primers designed to anneal either side of an ARE in the *KLK2* enhancer region (For- 5' TTGAAAGCAGACCTACTCTGGA-3'; Rev- 5'CTGGACCATCTTTTCAAGCAT-3') (33).

RT PCR

LNCaP cells were seeded at a density of $0.7x10^6$ cells/ml in 24 well plates with charcoal stripped FCS/ RPMI media for 16 hours before transfection with either 200ng of pCDNA3.1-CRKL or the control vector (empty pCDNA3.1) as a negative control. 24 hours later, cells were either treated with 10nM of mibolerone (MB), Casodex (1μM) or its carrier ethanol for a further 24 hours before harvesting. RNA was recovered using the RNAqueous-Micro kit (Ambion) following the manufacturer's recommendation. The RNA was quantified using a Nanodrop 2000 micro-sample quantitator. 1μg of total RNA from each sample was reverse transcribed using the One Step RT-PCR kit from Qiagen following the manufacturer's recommendation. Expression of human PSA was measured semi-quantitatively by PCR using primer pairs: Forward- 5'-TTGTCTTCCTCACCCTGTCC-3' and Reverse-5'TCACGCTTTTGTTCCTGATG-3' for 25 cycles at 94˚C for 1min, 58˚C for 45 seconds and 72˚C for 1min. GAPDH was used as a loading control and amplified by PCR using the primer pairs: Forward- 5'-GTGAAGGTCGGAGTCAACG-3' and Reverse-5'-GGTGAAGACGCCAGTGGACTC-3' for 30 cycles at 94˚C for 45 sec, 60˚C for 45 seconds and 72˚C for 1min.

siRNA targeted knockdown

LNCaP cells were seeded in androgen depleted media at a density of $7x10^5$ cells/ml in a 24 well plate for 24 hours and transfected with 40nM final concentration of siRNA AR (Dharmacon) or 200nM final concentration of siRNA CRKL (Dharmacon), SRC-1e (Dharmacon) and scrambled siRNA (Ambion) using Nanofectamin (PAA). Cells were incubated for 72 h with siRNA for efficient knockdown. For reporter assay data, cells were transfected with the relevant siRNA together with TAT-GRE-EIB-Luc and pdmLacZ-β-Gal for 48 hours before addition of ligand for a further 24 hours before harvesting.

Western blotting

All cell extracts were prepared at a concentration of 30μg per well in SDS-PAGE loading buffer and loaded on to Novex 4-20% Tris-Glycine Gels (Invitrogen). Under denaturing conditions, proteins were separated by gel electrophoresis and transferred onto nitrocellulose membrane using a semi-dry blotting apparatus (Trans-Blot SD Semi-Dry, Bio-Rad). The membranes were blocked in TBS containing 5% non-fat milk for 1 hour before incubating with primary antibodies for 1 hour at room temperature. Antibodies used were mouse anti-CRKL (Cell Signalling), rabbit anti CRKL (Santa Cruz), rabbit anti- AR (N20) (Santa Cruz), rabbit anti SRC-1e (Cell Signalling), rabbit anti phospho CRKL (Tyr 207) (Cell Signalling), rabbit anti ERK (Cell Signalling) and rabbit anti Phospho ERK (Cell Signalling). After subsequent membrane washing, detection was carried out using the horseradish peroxidase conjugated anti-mouse, anti-rabbit, or mouse IgG secondary antibody (1:5000) (Jackson Immuno Research) and incubated for 1 hour at room temperature. Following further washes, proteins were visualised using the enhanced chemiluminescence detection system (Amersham).

KEY RESEARCH ACCOMPLISHMENTS

We have completed all the questions for Task 1:

- In vivo screening in tumor-bearing mice (human prostate cancer xenografts) by phage peptide libraries have been successfully completed.
- Specific phage peptides have been identified and isolated from the tumor-bearing mice injected with the phage libraries.
- Few of the phage peptides have been characterized for their binding and inhibition properties on prostate cancer cell lines including DU145, PC-3, and LNCaP. Also, the phage peptides have been tested in the tumor-bearing mice.
- Localization and immunohistochemical analysis have been performed for some of the specific phage peptides on tissue samples from the tumor-bearing mice and prostate cancer cell lines.
- We have identified a new molecular complex for prostate cancer— the CRKL and beta1 integrin complex. This work has been published in a peer-review journal *PNAS* (Mintz et al., 2009, please see appendix)
- A review article has been accepted for publication in a peer-review journal *Cell Cycle* (Ozawa et al., 2010, please see appendix) regarding the work performed in Task 2.

We have completed all the questions for Task 2:

- Specific tumor-homing phage peptides have been identified and analyzed by protein databases (NCBI) for biologically relevant receptor leads for prostate cancer.
- A specific peptide was selected based on biochemical and functional assays. Affinity chromatography and biochemical cell fraction was used to identify the corresponding receptor.
- The corresponding receptor has been identified as being CRKL. We have characterized CRKL in the context of prostate cancer. Specifically, we have demonstrated that CRKL functionally interacts with the AR by several methods including immunofluorescence, immunoprecipitation, siRNA knockdown, and reporter assays.
- We have demonstrated that CRKL-AR complex can transactivates a prostate specific gene—PSA (prostate specific antigen) and the over-expression of CRKL in prostate cancers cells increases PSA expression level.
- We have shown that the CRKL-AR complex can operate independent of hormone stimulation through an alternative cell signalling pathway such as the ERK/MAPK pathway via EGF activation.
- We have prepared and submitted the work that was all accomplished in Task 2 to a peer review journal for publication (Reebye et al., 2009, please see appendix).

REPORTABLE OUTCOMES

- A paper has been published in a peer-review journal *PNAS* (Mintz et al., 2009, please see appendix).

- A review article has been accepted in a peer-review journal *Cell Cycle* (Ozawa et al., 2010, please see appendix).

- A second manuscript has been prepared and submitted for publication for 2009 (Reebye et al., 2009, please see appendix).

- A travel and presentation award at the Young Prostate Researchers Symposium, 2009, Institute Cancer Research (Cambridge, UK). The title: Activation of AR signalling by a ternary complex.

- Invited to be on a panel to review for the European Commission's 7th Framework Programme for Research (November 2009), "Translational research on cancers with poor prognosis".

CONCLUSIONS

We have identified a couple of new receptor-ligand complexes in prostate cancer. The association between β_1 integrin/CRKL and AR/CRKL are novel discoveries and this is the first report of such findings. The results and the completion of Task 1 in the first annual report where we have characterized the interaction between β_1 integrin/CRKL has culminated into a publication in a peer-review journal *PNAS* (Mintz et al., 2009). In addition, a review article regarding the work published in *PNAS* has been accepted in a peer-review journal, *Cell Cycle* (Ozawa et al., 2010, in press). For Task2, we have successfully performed all the necessary experiments to functionally characterize and validate another new molecular complex—CRKL/AR in prostate cancer. The culminating results from Task 2 has been prepared and submitted for a publication in a peer-review journal for 2009 (Reebye et al., 2009). We are very grateful for the funding from the Department of Defense (DOD). This work could not have been possible without the generous funding from DOD. Our work will have important impact on the prostate community as these findings are novel and potential new therapeutic targets to fight against prostate cancer.

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APPENDICES

- A article published in a peer-review journal *PNAS*, Mintz et al., 2009 entitled “An unrecognized extracellular function for an intracellular adapter protein released from the cytoplasm into the tumor microenvironment”.

- A review article published in a peer-review journal *Cell Cycle*, Ozawa et al., 2010 (in press) entitled “Cracking the code for compartment-specific dual functionality proteins in cancer: CRKL as another example”.

- A submitted article for publication, Vikash et al., 2009 entitled “Cooperation between the androgen receptor and a non-steroid signalling adaptor protein in prostate cancer cells”.

An unrecognized extracellular function for an intracellular adapter protein released from the cytoplasm into the tumor microenvironment

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Mammalian cell membranes provide an interface between the intracellular and extracellular compartments. It is currently thought that cytoplasmic signaling adapter proteins play no functional role within the extracellular tumor environment. Here, by selecting combinatorial random peptide libraries in tumor-bearing mice, we uncovered a direct, specific, and functional interaction between CRKL, an adapter protein [with Src homology 2 (SH2)- and SH3-containing domains], and the plexin-semaphorin-integrin domain of β_1 integrin in the extracellular milieu. Through assays in vitro, in cellulo, and in vivo, we show that this unconventional and as yet unrecognized protein–protein interaction between a regulatory integrin domain (rather than a ligand-binding one) and an intracellular adapter (acting outside of the cells) triggers an alternative integrin-mediated cascade for cell growth and survival. Based on these data, here we propose that a secreted form of the SH3/SH2 adaptor protein CRKL may act as a growth-promoting factor driving tumorigenesis and may lead to the development of cancer therapeutics targeting secreted CRKL.

cancer | CrkL | integrin | phage display

Cell membranes have evolved as a tight and compartmentalized, but dynamic, interface between intracellular and extracellular contents (1, 2). To maintain such homeostasis, transmembrane receptors mediate bidirectional signaling across the cell surface through a complex spatial and temporal organization (3, 4). Thus, protein location in signal transduction is central to specificity of cellular responses (2, 5, 6). For example, cell surface receptors such as integrins undergo conformational changes elicited through ligand binding to enable a cross-talk with signal transduction cascades such as the MAPK pathways; conventional integrin ligands include ECM proteins that recognize integrin extracellular domains and cytoskeletal proteins that interact with intracellular domains (3–4, 7–9).

To gain insight into signal transduction across cell membranes in cancer, we set out to identify functional protein interactions in a tumor xenograft model; we reasoned that a combinatorial approach (10–14) in vivo might provide clues by emulating ligand–receptor binding in the context of the tumor microenvironment. Here, we show a specific interaction between the intracellular signaling protein CRKL and a regulatory (rather than ligand-binding) β_1 integrin extracellular domain. Surprisingly, we found that CRKL targets the plexin-semaphorin-integrin (PSI) domain of β_1 integrin chain located outside of the cell and promotes cell growth and survival. These results indicate an unrecognized integrin-mediated outside-in function for intracellular mediators, such as Src homology 2 (SH2)- and SH3-containing proteins, in activating proliferative pathways.

Results

Combinatorial Selection in Vivo Yields Tumor-Homing Peptides. We administered a phage library (12, 14, 15) i.v. into nu/nu (nude) mice bearing human DU145-derived prostate cancer xenografts and recovered tumors after 24-h circulation. We recovered an enriched population of tumor-targeting phage (Fig. 1*A*) and individual phage clones (Fig. 1*B*). The dominant peptide (YRCTLNSPFFWEDMTHECHA) was functionally characterized; we evaluated its tumor-targeting specificity in vivo in tumor-bearing mice. After i.v. administration of YRCTLNSPFFWEDMTHECHA-phage, we observed marked homing to tumors (insertless phage served as a negative control) with barely detectable phage localization in several control organs (Fig. 1*C*). We also in vitro-targeted DU145 cells with an aqueous-to-organic phase separation assay (16) and a phage-based immunofluorescence assay (Kaposi Sarcoma cells; KS1767); consistently, YRCTLNSPFFWEDMTHECHA-phage bound to tumor cell surfaces to a greater extent than negative control phage (Fig. 1 *D* and *E*). We next evaluated the internalization of the peptide by fusing a proapoptotic motif (13, 17–19) to the tumor-homing sequence. We found targeted cell death relative to controls (Fig. 1 *F* and *G*), indicating that YRCTLNSPFFWEDMTHECHA mediates ligand-directed internalization. These results show that YRCTLNSPFFWEDMTHECHA targets tumor cells and enables internalization.

A Tumor-Homing Peptide Sequence Mimics a Regulatory Integrin Extracellular Domain. To determine whether the peptide sequence mimics a native protein, we performed a similarity search of YRCTLNSPFFWEDMTHECHA and other selected peptide sequences. By using BLAST followed by protein alignment, we found that the peptides resembled sequences present on β_1 integrin. Unexpectedly, the dominant sequence YRCTLNSPFFWEDMTHECHA had similarity to the PSI domain (residues 26–78) of the β_1 integrin; moreover, we found that other selected peptides also appeared within the same region. We then asked whether the

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The authors declare no conflict of interest.

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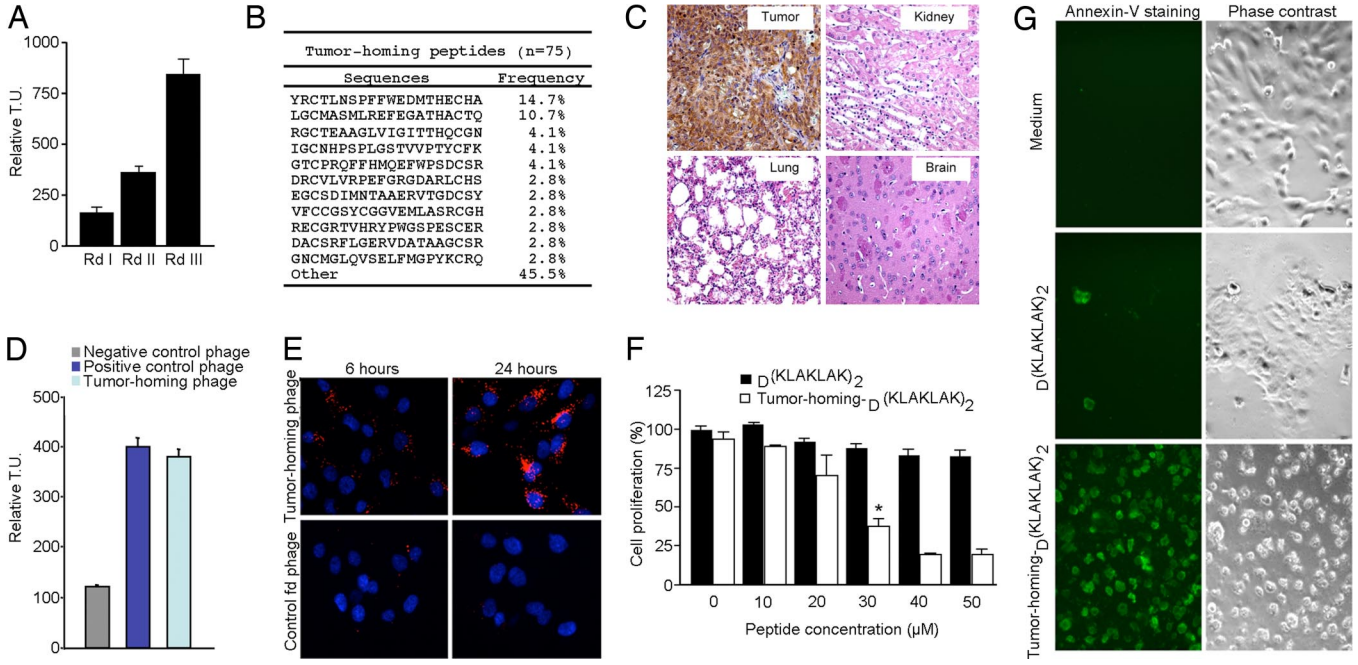

Fig. 1. Targeting tumor xenografts reveal a β_1 integrin mimic peptide. (*A*) Isolation of tumor-homing phage by screening of a random cyclic $X_2CX_{14}CX_2$ phage library on DU145-derived prostate cancer tumor-bearing mice. Bars represent mean ± SD from triplicate plating. (*B*) Peptide sequence analysis from the third round of in vivo selection. Seventy-five peptide sequences were recovered and analyzed. Frequency reflects the number of phage from the total recovered pool. (*C*) Targeting specificity for the phage displaying the peptide YRCTLNSPFFWEDMTHECHA in the prostate tumor-bearing mice. Antiphage staining by peroxidase reactivity was found in the tumor but not in control organs. Representative images are shown. (*D*) The YRCTLNSPFFWEDMTHECHA-displaying phage binds to the cell surface on DU145 cells. A phage clone displaying the sequence RGD-4C (12) served as a positive control, and fd-tet (insertless) was a negative control. Bars represent mean ± SD from triplicate plating. (*E*) Immunolocalization of tumor-homing phage on the cell surface of nonpermeabilized KS1767 cells. (*F* and *G*) Synthetic peptide YRCTLNSPFFWEDMTHECHAGG-$_D$(KLAKLAK)$_2$ (30 μM) enables internalization by DU145 cells. Cell viability with WST-1 reagent (*F*) and an anti-annexin-V FITC-labeled antibody (*G*) confirmed targeted cell death by the tumor-homing peptide internalization. Representative images are shown. *, $P < 0.001$ at 30 μM vs. $_D$(KLAKLAK)$_2$ alone (Student's *t* test). (Magnifications: *C*, ×200; *E*, ×400; *G*, ×200.)

similarity of the peptide YRCTLNSPFFWEDMTHECHA was specific for the PSI domain of the β_1 integrin or common to other known integrin β chains. After fit analysis and molecular modeling, we concluded that the homology between YRCTLNSPFFWEDMTHECHA and the PSI domain of β_1 integrin was indeed the best alignment (Fig. S1 *A–C*).

A Cytoplasmic Adapter Serves as a Receptor for the PSI Domain-Like Tumor-Homing Peptide. The integrin PSI domain has been characterized for regulatory activity (20–22). Given our results, we hypothesized that this domain might also function as a ligand-receptor binding site in β_1 integrins and set out to purify extracellular proteins that might bind to this region. We used affinity chromatography to identify binding partners to YRCTLNSPFFWEDMTHECHA (Fig. 2 and Fig. S2). We precleared a DU145-derived cell extract in a control peptide column, passed the precleared extract through the YRCTLNSPFFWEDMTHECHA column, and performed an acidic elution to detect a specific gel band corresponding to an ≈40-kDa protein (Fig. 2*A*). Mass spectrometry identified such protein as CRKL (ref. 23 and Fig. S1*D*); we validated the purified protein by immunoblotting (IB) with an anti-CRKL antibody (Fig. 2*B*). Next, we constructed recombinant His-tag proteins (rCRKL) and 3 corresponding domains [rCRKL-SH2, rCRKL-SH3 (N), and rCRKL-SH3 (C)] for binding assays and found that the peptide bound to rCRKL preferentially through the 2 SH3 domains of the protein. In contrast, little to no binding was detected through the SH2 domain or to control proteins (Fig. 2*C*); several controls with unrelated sequences had no binding. We also performed binding studies with a synthetic peptide of the β_1 integrin PSI domain (NSTFLQEGMPTSA; residues 50–62), which overlaps the selected sequences in the native PSI (Fig. S1 *A–C*); again, NSTFLQEGMPTSA bound to rCRKL, rCRKL-SH3 (N), and rCRKL-SH3 (C) (Fig. S2*A*). Moreover, we generated cyclic and linear peptides from the PSI domain and found that disulfide bonds are not essential for binding to CRKL (Fig. S3*A*). Finally, we showed that the interaction between SH3 (C) and the tumor-homing peptide is specifically inhibited by the synthetic peptide and by the phage clone itself (Fig. 2*D*). These findings show that the tumor-homing peptide and β_1 integrin-specific PSI domain-mimic, YRCTLNSPFFWEDMTHECHA, targets SH3 domains of CRKL. Other studies have shown that CRKL-SH3 domains homodimerize (24). The tumor homing peptide targets the monomeric and dimeric forms of CRKL-SH3 (data not shown), suggesting that the peptide-binding site is not involved in CRKL dimerization; however, the exact mechanism of peptide interaction with SH3 domains and competition with the PSI domain warrant additional study. Furthermore, SH3 domains bind to PXXP and non-PXXP motifs in addition to motifs with a single Pro (25–29). Because YRCTLNSPFFWEDMTHECHA does not contain any such motifs, we used site-directed mutagenesis to evaluate the binding attributes of the tumor-homing peptide. Binding to the CRKL-SH3 (C) domain depended on the Pro and the cyclic Cys-Cys bridge in the peptide (Fig. 2*E*); insertless phage and unrelated peptides were controls. We also performed mutational analysis of CRKL SH3 (C) and found the binding region to be between Gly-236 and Trp-277 (Fig. S3*B*). Both the tumor-homing phage and the PSI-derived phage (CNSTFLQEGMPTSAC) bind to rCRKL (Fig. S2*B*). Additional CRKL SH3 domain mutants (including domains that cannot bind PXXP or non-PXXP motifs) might yield insights into the binding of the tumor-homing peptide in future studies. By affinity chromatography, we established that CRKL can be purified from DU145 serum-free conditioned me-

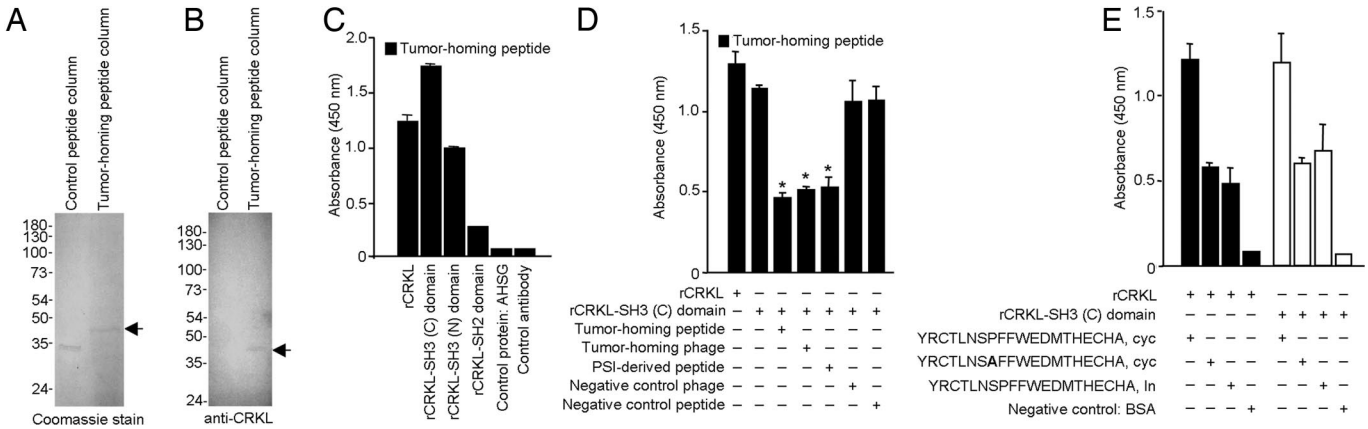

Fig. 2. Receptor identification and validation. (*A*) Receptor purification by peptide YRCTLNSPFFWEDMTHECHA affinity chromatography. A 40-kDa band was detected in the Coomassie blue-stained gel and was excised for protein sequencing. (*B*) An anti-CRKL antibody confirmed the identity of the 40-kDa band by immunoblot. (*C*) The recombinant His-tag CRKL (rCRKL), rCRKL-SH3 (N) domain, and rCRKL-SH3 (C) domain bind to the tumor-homing peptide YRCTLNSPFFWEDMTHECHA. $P < 0.001$ vs. controls (Student's *t* test). (*D*) The binding activity of the tumor-homing peptide to rCRKL-SH3 (C) domain is inhibited by the tumor-homing peptide YRCTLNSPFFWEDMTHECHA, the PSI-derived (NSTFLQEGMPTSA) peptide, or the tumor-homing phage displaying YRCTLNSPFFWEDMTHECHA. Bars represent mean ± SD from triplicate wells. *, $P < 0.001$ vs. rCRKL alone or rCRKL-SH3(C) (Student's *t* test). (*E*) Binding properties of the rCRKL-SH3 (C) to the tumor-homing peptide. A Pro → Ala mutant is shown in bold. cyc, cyclic; ln, linear. $P < 0.02$ vs. Pro → Ala cyc peptide (Student's *t* test).

dium and that a control column with a mutant tumor-homing peptide no longer bound to CRKL at detectable levels (Fig. S2*C*).

By reciprocal coimmunoprecipitation (IP) assays with membrane fractions, we showed that CRKL and β_1 integrin form a complex at the membrane; in contrast, control antibodies raised against membrane receptors (anti-IL11R, anti-EGFR, anti-β_3 or -β_5 integrins) had no association with CRKL or β_1 integrin (Fig. 3*A*). Finally, the interaction between CRKL and β_1 integrin is inhibited (concentration-dependent) by a recombinant PSI domain (IC_{50} = 20 nM; Fig. 3*B*); consistent with the co-IP, control integrins showed no binding (Fig. 3*A*).

CRKL Can Localize Outside of Cells. Molecular imaging showed colocalization of CRKL and β_1 integrin (Fig. 4 and Fig. S4), indicating a specific molecular interaction at the cell surface. Because the tumor-homing peptide binds DU145 cell surfaces, we tested the possibility that CRKL may also exist outside of the cell membrane. FACS analysis of nonpermeable DU145 cells showed surface labeling with an anti-CRKL antibody (Fig. 4*A*). We found cell-surface labeling by immunofluorescence and confocal imaging in cells treated similarly (Fig. S4*A*). We also evaluated ultrastructural localization by scanning and transmission electron microscopy (TEM) with intact membranes. These imaging approaches yielded only modest CRKL cell-surface labeling but remain consistent with nonenhanced fluorescence detection methods (Fig. 4 *B* and *C*). Finally, we used biochemical approaches to confirm cell membrane localization of CRKL: cell surface labeling by biotinylation (Fig. 4*D*) and detergent membrane fractionation (Fig. S4*B*). By either method, we found CRKL present on the cell membrane (Fig. 4*D* and S4*B*). Antibodies against unrelated intracellular proteins served as controls (Fig. S4*B*). These results show that CRKL, in addition to its cytoplasmic location, is present on the surface.

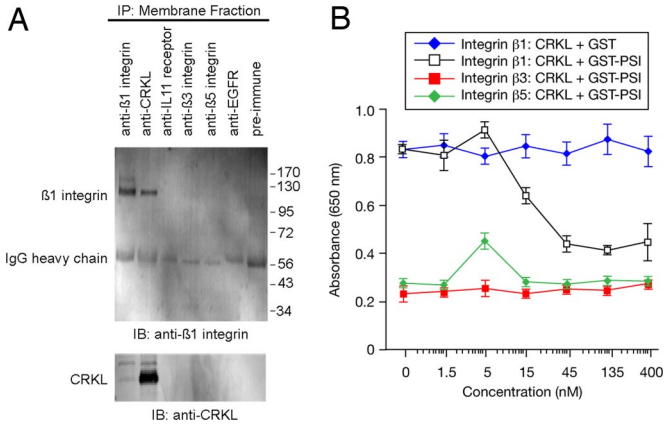

Fig. 3. Interaction between CRKL and β_1 integrin. (*A*) The association of CRKL with β_1 integrin by reciprocal coIP from a DU145 membrane fraction with either anti-CRKL antibody or anti-β_1 integrin antibody. The following unrelated antibodies served as negative controls: anti-IL11R, anti-EGFR, anti-β_3, anti-β_5, and preimmune serum. (*B*) A concentration-dependent inhibition of CRKL binding to β_1 integrin by recombinant GST-PSI protein. The integrins $\alpha v\beta_3$ and $\alpha v\beta_5$ were used as controls. Mean ± SD from triplicate wells are shown.

Functional Studies and Transport Mechanisms. There are two potential explanations for finding extracellular CRKL: active transport by a secretory mechanism and/or passive release of intracellular contents caused by cell death. Because CRKL lacks a classic transmembrane domain, we evaluated whether CRKL is secreted from tumor cells (Fig. 5 and Fig. S5); we found that DU145 cells cultured in a serum-free medium do secrete unphosphorylated CRKL. In contrast, CRKL was not detected in controls as shown by IP either with anti-CRKL or control antibodies (Fig. 5*A*). To assess the generality of these data, we examined a panel of tumor cell lines in serum-free media and found that they also secrete unphosphorylated CRKL (Fig. 5*B*), indicating that this phenomenon is not cell type specific and highly depends on the tumor microenvironment for surface binding. Next, we sought to determine whether CRKL secretion had detectable effects on cell proliferation and migration. To show specificity, we added an anti-CRKL-neutralizing antibody to the serum-free medium of DU145 cells. We found that the anti-CRKL antibody does neutralize extracellular CRKL and reduced cell proliferation and cell migration; preimmune serum or control antibodies did not yield detectable effects on cell proliferation or migration (Fig. S6 *A* and *B*). To further understand the role of CRKL in tumor cells, we silenced CRKL with siRNA; again, we found reductions in cell proliferation, adhesion, and migration with CRKL reduction (Fig. S6 *A*–*C*). As an additional control, we showed that the decrease in proliferation is rescued by exogenous CRKL (Fig. S6*D*); only background apoptosis (≈1%) was detected in CRKL siRNA-treated cells or cells in serum-free medium. Finally, we found, in the

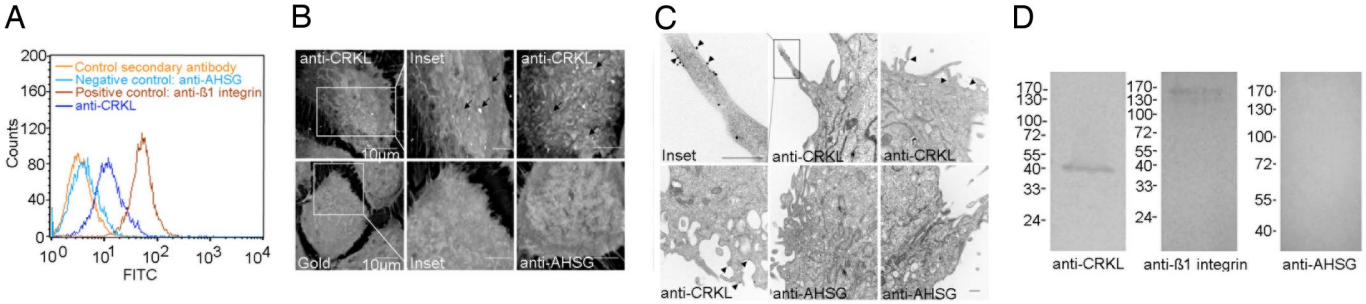

Fig. 4. CRKL is localized at the cell surface. (*A*) Flow cytometry analysis of CRKL on DU145 cells. Immunolabeling was performed with monoclonal anti-CRKL, anti-β_1 integrin, and anti-AHSG antibodies. (*B*) Scanning electron microscopy of CRKL localization. Scale bar, 5 μm unless specified (arrows indicate CRKL-gold conjugated labeling). (*C*) TEM of CRKL showing individual CRKL-gold particles on the cell surface (arrowheads). The DU145 cells used in *B–D* were fixed under conditions in which cell plasma membranes were retained intact. Scale bar 500 nm. Representative images are shown. (*D*) (*Left*) Cell-surface biotinylated DU145 cells exhibit a surface location of CRKL. (Negative controls *Center* and *Right*). An anti-CRKL polyclonal antibody was used in (*B–D*).

CRKL siRNA-treated cells, reduced binding of the tumor-homing phage to the cells, indicating that tumor-homing phage might bind through secreted CRKL (Fig. S5*C*). Given that CRKL lacks a hydrophobic N-terminus for secretion via the endoplasmic reticulum and Golgi-dependent pathway (30), we tested whether secretory inhibitors might prevent cell release of CRKL. We showed that the inhibitors brefeldin A and thapsigargin do not inhibit CRKL secretion (Fig. 5*F*); in contrast, we found that glybenclamide, an inhibitor of ABC transporters, prevents CRKL release (Fig. S5*C*). There are at least 4 processes through which a protein can be secreted without a classic signal peptide (31–33). Our data are consistent with CRKL secretion by a nonclassical export pathway via ABC transporters. Other growth factors use ABC transporters (34, 35), suggesting a plausible working hypothesis. These results do not exclude the possibility that cell death caused by clonal selection or postcytotoxics could also generate extracellular CRKL in the tumor microenvironment. We thus designed binding assays to detail the biochemical interactions among CRKL, β_1 integrins, and the PSI-mimetic. With rCRKL (GST and His-tag), our results suggest that CRKL can oligomerize (Fig. S7), perhaps through the SH3 domains (24, 36, 37).

Furthermore, addition of exogenous CRKL leads to the phosphorylation of proteins in the MAPK and integrin-mediated signaling cascades, suggesting direct activation of migratory and proliferative pathways (Fig. S8).

CRKL-Mediated Ligand-Directed Tumor Targeting. We next evaluated the targeting of CRKL-binding phage in vitro (Fig. 6*A*) and in tumor-bearing mice. First, we produced constructs displaying the selected CRKL-binding peptide or control (mutant or scrambled) peptides. Targeting was tested in human tumor xenografts [DU145 (Fig. 6*B*) and KS1767 (Fig. S9*A*)] and an isogenic mouse tumor [EF43-*FGF4* (Fig. S9*B*)]. We observed marked and specific tumor homing after i.v. administration of CRKL-binding phage; in contrast, controls showed no tumor localization (Fig. 6 and Fig. S9). Further, we showed that homing is inhibited whether CRKL-binding phage is preincubated with rCRKL before administration to tumor-bearing mice (Fig. 6*C*). Notably, the tumor targeting of CRKL-binding phage was at least ≈10-fold higher relative to that of RGD-4C phage (10, 12, 15–17, 38), used as positive control. We then conducted a pilot preclinical trial with 4 size-matched cohorts of tumor-bearing mice (Fig. 6*D*). Animals received the following: (*i*) synthetic PSI domain-mimic peptide, (*ii*) synthetic PSI domain-mimic peptide fused to a proapoptotic motif (13, 17–19) to induce targeted apoptosis upon receptor-mediated internalization, (*iii*) synthetic proapoptotic motif, or (*iv*) vehicle. Peptides were administered at equimolar concentrations. The PSI domain-mimic pep-

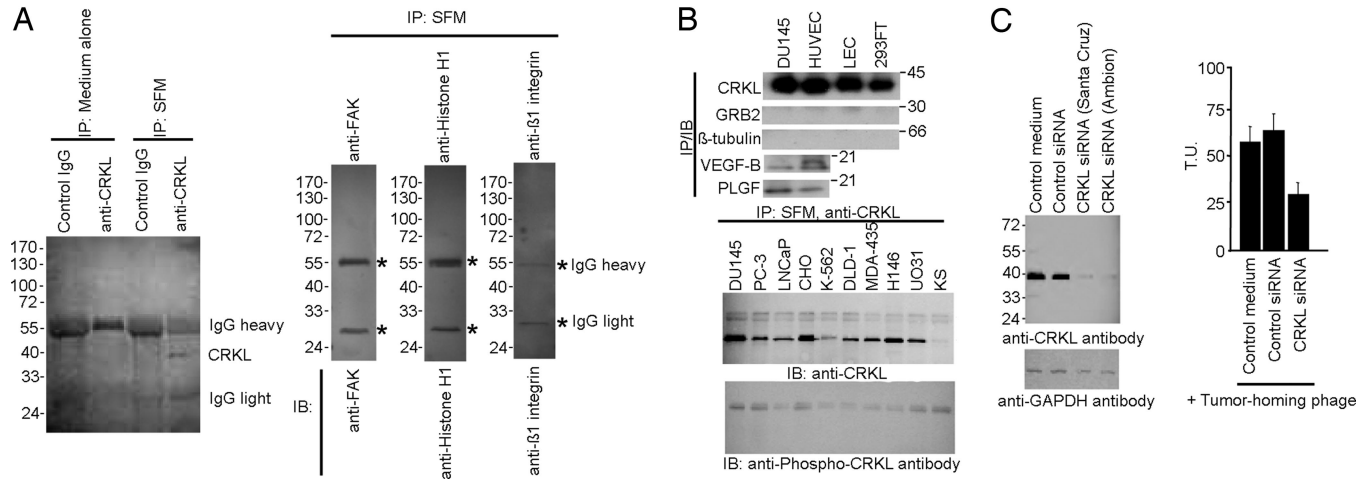

Fig. 5. CRKL secretion and cell signaling. (*A*) CRKL is secreted by DU145 cells cultured in serum-free medium (SFM) after 24 h as shown by detergent-free IP with an anti-CRKL antibody. Control antibodies (anti-FAK, anti-histone H1, and anti-β_1 integrin) do not pull down their corresponding proteins in the cultured SFM. (*B*) (*Upper*) CRKL is secreted in several cell lines. IP and IB for GRB2 and β-tubulin served as negative controls; Placental growth factor (PLGF) and VEGF-B served as positive controls. (*Lower*) Other cell types cultured in SFM appeared to secrete only the unphosphorylated form of CRKL. (*C*) siRNA silencing of CRKL reduced tumor-homing phage binding to DU145 cells. SEM from triplicate wells is shown.

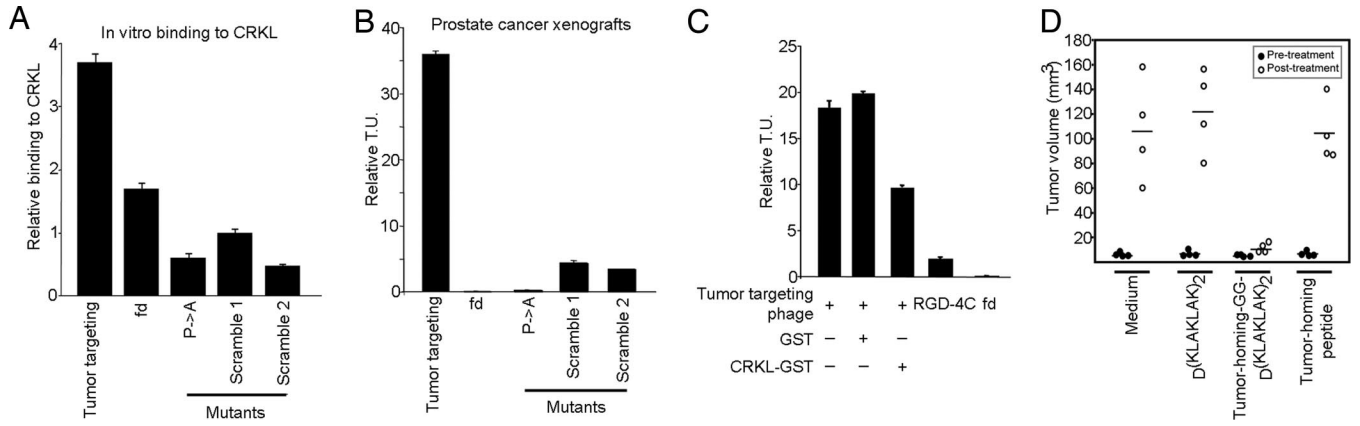

Fig. 6. Tumor targeting. (*A*) In vitro phage binding of tumor-homing or controls (insertless, mutant, or scrambled) phage on rCRKL. Results are expressed as mean ± SEM of triplicate wells. (*B*) In vivo homing of targeted or control phage constructs in mice bearing prostate tumor xenografts. Tumor-homing phage localized to tumors preferentially compared with controls. Representative data from 2 independent experiments are shown. Bars represent mean ± SD. Tumor targeting, YRCTLNSPFF-WEDMTHECHA; fd, fd-tet phage (negative control); P->A, YRCTLNSAFFWEDMTHECHA; Scramble 1, YRFCTSPFHEWHLENTDMCA; Scramble 2, YRECTDSPHEFHLWNT-MCAF (bold indicates residue mutations). (*C*) Targeting inhibition in tumor-bearing mice. The tumor-homing phage was preincubated with control GST or recombinant GST-CRKL before administering to nude mice bearing size-matched human tumors (DU145-derived). Inhibition was observed in the pretreated tumor-homing phage by recombinant CRKL. Results from 2 independent experiments are shown. (*D*) Tumor treatment with a synthetic tumor-homing proapoptotic peptidomimetic. Cohorts of size-matched nude mice bearing human prostate cancer xenografts (DU145-derived) were used. Markedly reduced tumor growth was observed in tumor-bearing mice treated with synthetic tumor-homing proapoptotic peptidomimetic YRCTLNSPFFWEDMTHECHA-GG-$_D$(KLAKLAK)$_2$. Equimolar amounts of YRCTLNSPFFWED-MTHECHA or $_D$(KLAKLAK)$_2$ showed no differences in tumor volume compared with untreated animals. $P < 0.001$ (Student's *t* test).

tide had no detectable effect on tumor growth, whereas the targeted proapoptotic motif inhibited tumor growth.

Discussion

Consistent with the data presented here, it is likely that extracellular CRKL plays an as-yet-unrecognized role in the tumor microenvironment by triggering cell proliferation and migration. Because intracellular CRKL is also phosphorylated after addition of exogenous rCRKL, one might speculate that extracellular CRKL can function as an autocrine or paracrine factor within tumors. Our results establish an unusual connection between signaling and cell adhesion molecules, in which their relationship at the cell surface can trigger signaling events from the extracellular milieu. Based on the "switchblade" structural model for integrin activation (39) we propose a working model in which extracellular CRKL activates integrins through binding to the β_1 integrin PSI domain (Fig. S10). We show that intracellular unphosphorylated CRKL is secreted by a nonclassical active transport and/or released through cell death into the microenvironment (step 1), where its SH3 domains bind specifically to the PSI domain of the β_1 integrin on the tumor cell surface (step 2). Upon binding, the β_1 integrin changes conformation from a bent to extended (active) form and thereby triggers downstream phosphorylation of target proteins in the integrin-mediated pathway (steps 3 and 4) and/or MAPK pathway (steps 5–7), affecting tumor cell migration and proliferation (step 8). Precedent for intracellular molecules such as nuclear proteins (40, 41), transcription factors (42), and stress-response chaperones (13, 43, 44) on the cell surface has been reported. Ligand–receptor interactions between signaling molecules and surface receptors within the extracellular environment may have general biological significance.

Materials and Methods

Reagents. Anti-CRKL (Santa Cruz, Cell Signaling,Epitomics, or Upstate Biotechnology), antiphospho-CRKL (Cell Signaling), anti-β_1 integrin (Chemicon or BD), anti-IL11R (Santa Cruz), anti-β_3 and anti-β_5 integrins (45), anti-EGFR (46), anti-grb2 (Santa Cruz), anti-α_6 integrin (Chemicon), anti-fetuin A/α_2-Heremans-Schmid glycoprotein (AHSG; R&D Systems), preimmune serum (Jackson), anti-His (Santa Cruz), anti-GST (Santa Cruz), and anti-GAPDH (Ambion) were used. Peptides were synthesized to our specifications (AnaSpec). Nude mice were purchased (Harlan), and tumors were generated as described (13, 47). The Institutional Animal Care and Use Committee at the University of Texas M. D. Anderson Cancer Center approved animal experiments.

Cell Culture. Cell lines were purchased (ATCC) unless otherwise specified. Human lung epithelial cells and human umbilical vein endothelial cells were purchased and cultured in EGM-2 (Cambrex). Tumor cells were maintained in DMEM-high glucose (GIBCO) supplemented with 10% FBS, penicillin, streptomycin, and 4 mM glutamine (ICN) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Phage Display Library Selection. Screenings were performed as described (12–15). A random phage library displaying an insert with the general arrangement $X_2CX_{12}CX_2$ (C, cysteine; X, any residue) was administered (tail vein) into tumor-bearing nude mice and allowed to circulate for 24 h. Mice were placed under deep anesthesia, tumors were excised and weighed, and the bound phage population was recovered and processed.

Affinity Chromatography and Mass Spectrometry. Peptide affinity columns were made by 1-ethyl-3-(3-dimethylaminopropyl)carboiimide and diaminodipropylamine immobilization resin (Pierce). Cell extracts were prepared and first passed through a nonspecific control peptide column followed by the tumor-homing peptide column. Columns were washed, eluted with Gly (pH 2.2), analyzed by SDS/PAGE, and stained with Coomassie blue. Affinity purification of CRKL from serum-free conditioned medium was performed and confirmed with recombinant GST-tag fusion protein expressing tumor-homing or mutant control peptide (Pro → Ala). Serum-free conditioned medium (200 mL at 48-h culture) was concentrated for the affinity purification. Fusion proteins were coupled to GST-resin beads and poured into a column. The concentrated serum-free conditioned medium was added to the columns for an overnight incubation. After several washes, the bound CRKL was eluted for Western blot analysis. The blot was probed with anti-GST and anti-CRKL antibodies.

Cell Surface and Membrane Localization. Cell surface labeling with biotin (42), phage binding assays (16), and membrane fractionation (44) were performed as described. Immunofluorescence (IF), FACS, and TEM analyses were performed through standard protocols. For details, see *SI Text*.

Mutagenesis. Primers are summarized in Table S1. For details on recombinant proteins and scrambled and mutant tumor-homing phage, see *SI Text*.

siRNA. CRKL (mRNA accession no. NM_005207), β_1 integrin (mRNA accession no. NM_002211), and control siRNAs were purchased from Santa Cruz, Ambion, and Dharmacon. siRNA oligonucleotides are summarized in Table S2. Oligofectamine (Invitrogen) or DharmaFect (Dharmacon) were used to transfect siRNAs into DU145 cells (1–2 × 10^5 cells per well). Transfected cells were incubated for 48–72

h before processing, recovered, and lysed in the presence of protease inhibitors. Tumor-homing phage binding activity was examined in CRKL-silenced cells. For rescue experiments, up to 1.5 μg of recombinant CRKL was added to CRKL siRNA-transfected cells. Exogenous His-tag recombinant CRKL (400 ng/mL) or controls (EGF, 200 ng/mL; migration inhibitory factor, 300 ng/mL; phorbol 12-myristate 13-acetate, 300 ng/ml) were used in β_1 integrin-silenced experiments. Equal amounts of protein were loaded and resolved by SDS/PAGE followed by Western blot analysis with the appropriate antibodies. Cell proliferation assays were performed with WST-1 (Roche). Cell migration assays were performed in a Boyden chamber assay (Corning).

IP. Cells were synchronized for 24–48 h in RPMI serum-free 1640 medium, followed by centrifugation and sterile filtration and preabsorption to control antibodies. An equal volume of PBS was added to the recovered supernatant before IP with the appropriate antibodies. Brefeldin A, thapsigargin, and glybenclamide were used. Cells were incubated with the compounds in serum-free medium for 9 h before IP with the appropriate antibodies. No detergents were used in the IP. This protocol favors the finding of CRKL in a free soluble state rather than in vesicles.

Peptide Binding and Internalization. Peptides were coated on microtiter plates followed by blocking and washing. Mixtures were incubated, washed, and labeled with the appropriate antibodies. HRP-conjugated secondary antibodies were added followed by 3,3′–5,5′–tetramethylbenzidine (TMB) substrate (Calbiochem), and complexes were analyzed in an ELISA reader. To determine the inhibitory activity of the tumor-homing peptide, the PSI-derived peptide or the phage clone displaying the tumor-homing peptide were incubated with the rCRKL-SH3 (C) domain. Unrelated peptide sequences and insertless phage served as negative controls. Mixtures were incubated and added to wells coated with the tumor-homing peptide. After incubation, the wells were washed and labeled with the appropriate antibodies. For details and recombinant protein information, see *SI Text*.

Phage and Protein Binding Assays. Phage binding assays on purified proteins were carried out as described (19, 48). For a detailed description, see *SI Text*.

In Vivo Tumor Targeting and Inhibition. In vivo targeting experiments with phage were performed as described (10, 12, 13, 47). For details, see *SI Text*.

Therapy in Tumor-Bearing Mice. Tumor-bearing mice were size-matched and divided into individual cohorts (n = 4 mice per group). The tumor-homing peptide was synthesized fused with the proapoptotic motif $_D$(KLAKLAK)$_2$. Unconjugated peptide YRCTLNSPFFWEDMTHECHA or $_D$(KLAKLAK)$_2$ served as controls. Peptides were administered i.v. at 300 μg per mouse per week, and tumor volumes were measured (12, 13).

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Cracking the code for compartment-specific dual functionality proteins in cancer: CRKL as another example

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Bidirectional signaling across the plasma membrane is an essential process regulating cellular homeostasis. In normal tissues, it is often coordinated through external stimuli and cell surface receptors creating a balance between cell growth and death. In cancer, tumor cells develop genetic and epigenetic alterations often the result of activation of growth stimulatory pathways; such mechanisms permit uncontrolled cell proliferation and cell death prevention. Tumor microenvironment provides a key permissive and unique context to cancer progression. Indeed, experimental evidence suggests that the tumor microenvironment not only promotes growth but also becomes required for tumor cell survival.[1] Thus, understanding the tumor microenvironment is likely to provide insight into tumor biology and novel therapeutic strategies.

An increasing number of proteins with location-dependent function support the concept that single proteins may have multiple functions, a phenomenon recently termed "moonlighting".[2,3] For example, epimorphin and syntaxin 2 were identified as separate proteins functioning in different locations. Syntaxin 2 is an intracellular plasma membrane-associated protein involved in a number of membrane fusion processes, including vesicle docking and fusion[4]; epimorphin is an extracellular signaling molecule mediating epithelial tissue morphogenesis that was also subsequently shown to lead to alveolar hyperplasia and mammary adenocarcinoma when overexpressed in the mammary gland of transgenic mice.[5,6] While each molecule was originally identified and characterized separately, they were later determined to be encoded by the same gene with unique structural alterations leading to different cellular localization and function.[5] Notably, epimorphin/syntaxin2 functions in a coordinated fashion regulating its own localization for epithelial morphogenesis.[2] Several other examples have recently

emerged and include various nuclear proteins, transcription factors, and stress-response chaperones.[7] Thus, while many molecules have been shown to exhibit dual functionality,[2,7,8] these observations raise the question of whether other as yet unrecognized intracellular proteins may also function as coordinated autocrine regulators or whether there are other growth promoting signal transduction molecules within the tumor microenvironment that may meet these criteria.

Recently, to study tumor cell stimulating factors and functional protein-protein interactions at the cell surface in tumors, we employed an unbiased combinatorial approach targeting human prostate cancer xenografts in vivo.[7] Following serial rounds selection we identified a single peptide (YRCTLNSPFFWEDMTHECHA) with selective and robust systemic homing to tumor xenografts. We performed a protein similarity search with basic local alignment search tool (BLAST) and identified the plexin-semaphorin-integrin (PSI) regulatory domain of ß1 integrins as the native protein mimicked by the motif. As this specific targeting was likely mediated by a functional protein interaction,[9] we used in tandem affinity chromatography and mass spectrometry to identify the binding partner of the ß1 integrin specific PSI domain-mimic peptide. We showed that Crk-like (CRKL) is the protein binding to the targeting peptide. CRKL is an intracellular adapter protein localized in multiple cellular compartments that is intimately involved in cell signaling, and neural crest development.[10,11] It consists of one SH2 domain and two SH3 domains, allowing for the formation of hetero- and homodimeric proteins. Next, we validated that the PSI domain-mimic peptide indeed targets the SH3 domain of CRKL and that while the SH3 domains of CRKL do homodimerize,[12] the corresponding binding site is not involved in the dimerization.

Given the strategy by which the PSI domain-mimic and CRKL were identified, these data suggested that CRKL binds to the PSI domain of ß1 integrin on the extracellular surface. To support our interpretation, we used an array of molecular imaging techniques (i.e., FACS, immunocytochemistry, scanning electron microscopy and transmission electron microscopy) to confirm the presence of CRKL at the surface of prostate cancer cells. Moreover, detergent membrane fractionation experiments strengthened the imaging results and verified that CRKL exists in the intracellular and extracellular compartments of cultured tumor cells.

We reasoned that the existence of CRKL in the extracellular milieu indicates that the protein is either actively secreted from tumor cells or released upon cell death (or both). CRKL lacks a hydrophobic N-terminal signal peptide for endoplasmic reticulum or Golgi-dependent secretory pathways. Thus, we next evaluated the mechanism(s) by which CRKL may be secreted from tumor cells. Using secretory inhibitors (i.e., brefeldin A, thapsigargin and glybenclamide) we observed inhibition of CRKL release only with glybenclamide, an inhibitor inhibition of ABC transporters. While such data do not exclude a release of intracellular CRKL from tumor cell death, it does suggest that a nonclassical export pathway mediated by ABC transporters may actually secrete CRKL. These results are not unique: macrophage migration inhibitory factor and interlukin-1ß have also demonstrated such nonclassical ABC transporter dependent release from cells.[13,14]

Next, to explore a functional role(s), if any, of extracellular CRKL, we studied its migratory and proliferative effects on tumor cells. First, using a neutralizing anti-CRKL antibody, we observed a reduction in cell proliferation and migration compared to

controls. Silencing of CRKL with siRNA corroborated these results with a reduction in cell proliferation, migration, and adhesion. And addition of exogenous recombinant CRKL to the cell medium did rescue cellular proliferation in CRKL siRNA treated cells. Because intracellular CRKL is implicated in both MAP kinase and integrin-mediated pathways, we then searched for phosphorylated proteins in these two pathways.[15,16] Consistently, when recombinant CRKL was added to tumor cells in vitro, we found several phosphorylated proteins including paxillin, $p130^{Cas}$, Erk1, Erk2, and Elk1, and even CRKL itself. When exogenous recombinant CRKL was added to β_1 integrin siRNA-treated cells, reduced phosphorylation of Erk1 and Erk2 proteins was also found. This result indicates that ERK pathway activation by exogenous CRKL is dependent on the expression of β_1 integrin. Positive control mitogens activating the MAP kinase-dependent pathway and stimulating tumor cells show that the overall efficiency of the cell signaling machinery is maintained in CRKL siRNA-treated cells. These studies show that extracellular CRKL can activate integrin-mediated and MAP kinase pathways.

Together, the aggregate of these data demonstrate that CRKL serves as a dual function protein within the tumor microenvironment: as an intracellular adapter protein mediating cell signaling and as an extracellular binding partner for the regulatory PSI domain of ß1 integrin. One might speculate whether CRKL actually functions as an autocrine-type stimulatory factor in vivo (Fig. 1). We observed secretion of CRKL by multiple cell types in vitro; however, the strong selectivity of CRKL targeting in vivo supports a critical role of the tumor microenvironment in homing. Further study into the selectivity and mechanisms of CRKL action may bring greater understanding into such dual topology proteins and may ultimately yield novel therapeutic targets for

intervention. Moreover, in a broader context, these data also suggest that combinatorial library screenings in vivo can provide unbiased functional insight into the tumor microenvironment and may unveil additional proteins with compartment-specific roles in cancer.

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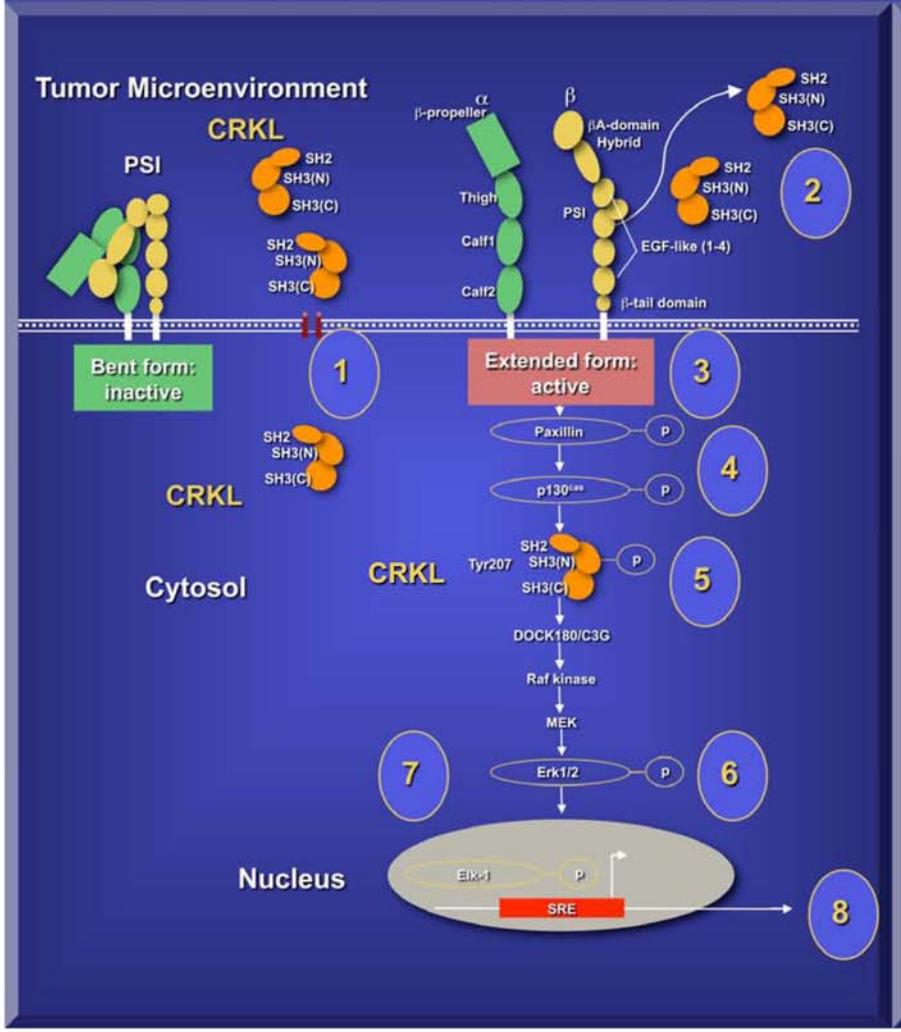
Figure Legend

Figure 1. A working model based on the switchblade integrin activation. A hypothetical pathway used by extracellular (secreted and/or released) CRKL to activate integrin-mediated MAPK cascade to promote cell proliferation and migration is shown. This figure is adapted from Reference 7 (Mintz PJ et al. An unrecognized extracellular function for an intracellular adapter protein released from the cytoplasm into the tumor microenvironment. Proc Natl Acad Sci U S A 2009, 106(7) 2182-2187. Copyright 2009 National Academy of Sciences, U.S.A.)

A Working Mechanistic Model for the Dual Function of CRKL in Cancer



A Working Mechanistic Model

1. CRKL secreted and/or released by cell death
2. CRKL SH3 domains bind to β_1 integrin PSI
3. β_1 integrin changes to active conformation
4. Target phosporylation : Paxillin & p130^{cas}
5. Intracellular CRKL phosphorylation
6. Activation of MAP kinase pathway
7. Nuclear transcription
8. Cell migration and proliferation

Ozawa et al., Figure 1

Cooperation between the androgen receptor and a non-steroid signalling adaptor protein in prostate cancer cells

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Key words: Androgen receptor, CRKL, Prostate cancer

ABSTRACT

The androgen receptor (AR) plays a critical role in prostate cancer development. Understanding the molecular mechanism of AR signalling in this setting is still at the forefront of prostate cancer research. Traditionally, the AR interacts with co-activators that are nuclear proteins; however, many non nuclear factors can also affect the AR at the transcriptional level. We report a cooperative interaction between CRKL (an intracellular signalling adaptor protein) and the AR. Although CRKL is defined as a cytoplasmic protein, we demonstrate by biochemical and genetic approaches that CRKL is also localised in the nucleus of prostate cancer cells and, when overexpressed, upregulates PSA level. A similar pattern of nuclear expression of CRKL is also found in advanced prostate cancer tissue biopsies. We show that CRKL forms a complex with the AR and SRC-1e and that CRKL is also present together with the AR at the enhancer region of the androgen regulated human kallikrein-2 gene. In addition, the functional interaction of CRKL and the AR appears to be partly driven by cross-talking with the ERK/MAPK signalling pathway. We have uncovered a novel role of CRKL with the AR and provide new insights into the interaction between an adaptor protein and the AR in prostate cancer cells.

Introduction

The androgen receptor (AR) is a member of the nuclear receptor superfamily and is important for the development of the prostate gland as well as the pathogenesis of prostate cancer as it is expressed to various degrees in virtually all primary prostate cancers (1-3). Mechanistically, the AR remains transcriptionally inactive in the cytoplasm of prostate epithelial cells via its interaction with heat shock proteins, chaperones and other regulatory elements (4, 5). Exposure to androgens causes a conformational change of the AR via its ligand binding domain (LBD) which changes its interaction dynamics with many of the coregulatory proteins. This results in nuclear translocation, receptor dimerisation and recruitment of the general transcriptional machinery at specific androgen regulated target genes (androgen responsive elements (AREs) (6). This is an orchestrated event in normal prostate epithelial cells where numerous co-regulators (activators and repressors) have been identified to be important in AR signalling (7). Since the AR is still functionally active in advanced prostate cancer despite androgen ablation therapies- advanced stages of the disease ultimately progress to an androgen independent stage that is resistant to current therapies (8, 9). It is likely that other signalling events are cross-talking with the AR signalling pathway; however, the underlying mechanism of this event and the factors involved still remains poorly characterised. A significant body of accumulated evidence however do implicate numerous mechanisms: They include gene amplification of the AR (10, 11); clonal selection of point mutations within the AR domains (12-16); an increased expression of enzymes involved in steroid production (17, 18); or alteration in the expression of coregulatory proteins (19, 20). It is suggested that overexpression of specific co-activators may facilitate cross-talk between the androgen and kinase signal transduction pathway thus allowing the AR to bypass its androgen dependent axis (9, 21). The different mechanisms put forward are necessarily not mutually exclusive as prostate cancer cells will adapt appropriately to its transformed androgen ablated environment for continued cell survival and proliferation.

We have identified that an intracellular adaptor protein - CRKL (Chicken tumour virus number 10 Regulator of Kinase-Like protein) interacts with the AR complex to regulate receptor activity at the transcriptional level. We demonstrate that CRKL may be an important regulator of the AR complex in advanced prostate cancer cells as its overexpression in the presence of growth factors is able to rescue the inhibitory effect of casodex on AR activity

RESULTS AND DISCUSSION

CRKL and the AR forms a functional complex

Here we studied a multi-functional adaptor cell signalling protein-CRKL, which is overexpressed in various types of diseases including prostate cancer (22, 23); and is used as a surrogate target for the kinase activity of BCR-ABL1 in chronic myeloid leukemic patients (24). CRKL is composed of a Src homology 2 domain (SH2) and two Src homology 3 domains (SH3) (22, 25). These domains are important for protein-protein interaction and intracellular cell signalling in transformed cancer cells (22, 26). Recently, CRKL was shown to activate the integrin-mediated ERK/MAPK pathway in prostate cancer cells (23). However, it is unknown if CRKL has any direct interaction with the AR signalling pathway. Since CRKL has the ability to translocate into the cell nucleus and interact with the transcription machinery (27) we investigated whether CRKL could play a role in AR mediated signaling in prostate cancer cells.

We first determined by co-immunoprecipitation whether CRKL and the AR formed a complex in PC3 prostate epithelial cells stably expressing wild type AR (PC3wtAR) (28) or in LNCaP cells which endogenously expresses the AR. We found that CRKL and the AR are present in a complex (Fig. 1A and B) and that CRKL is endogenously distributed in both the cytoplasmic and nuclear compartments of prostate cells with an observable increase in nuclear distribution following hormone stimulation (Fig. 1C) or following transient overexpression of CRKL (Fig. 1D). We then investigated the expression pattern of CRKL in human

prostate tissue biopsies from patients with different stages of prostate cancer. CRKL staining was more intense and appeared distinctly nuclear in patients with highly differentiated tumours (Gleason score 9) with stages T3A and T3B (where the tumour has extended beyond the prostate capsule) (Fig. 1E).

We next addressed whether the interplay between endogenously expressed AR and CRKL formed a functional complex at the transcriptional level in prostate cancer cells. A ChIP assay was performed in LNCaP cells either in the absence or presence of hormone to confirm that endogenous CRKL and the AR were both present on the enhancer region of the androgen regulated human Kallikrein 2 (KLK2) gene (Fig. 1F). KLK2 is widely known as prostate specific antigen (PSA) as they both share high homology (29). KLK2 also adds significant information when detecting prostate cancer (30-34). To understand the functional role of the AR-CRKL complex, a CRKL expression construct (pCDNA3.1-CRKL) was then used in an AR dependent transcription luciferase reporter assay in COS cells and PC3 cells. We found that overexpression of CRKL (Fig. 1G) enhanced hormone induced activity of the AR (Fig. 1H and I). To substantiate the reporter assays, we then measured the effects of CRKL overexpression on endogenous PSA levels in LNCaP cells treated with mibolerone or the AR inhibitor, casodex. There was a significant upregulation of PSA when CRKL was overexpressed relative to the control (empty vector transfection) (Fig. 1J). We also observed this effect in the absence of hormone stimulation and in cells treated with casodex suggesting that CRKL may act as a strong coactivator in LNCaP cells.

CRKL forms a complex with SRC-1e and the AR

Since the AR forms a large complex in the nucleus, we next determined whether CRKL also interacts with any known AR co-activators. We chose the most characterised activator of the steroid receptor superfamily, SRC-1e (a histone acetyltransferase and member of the p160 family of steroid receptor co-activator) (35). We found that CRKL and SRC-1e are in a complex with the AR when CRKL and SRC-1e were simultaneously overexpressed in LNCaP cells

(Fig. 2A), and that AR transcriptional activity was significantly enhanced (Fig. 2B). To gain greater insight into the transcriptional activity of the AR-CRKL complex, we tested what effect CRKL overexpression would have on an AR mutant (AR∆LBD) missing its ligand binding domain. This mutant AR contains only the activation function 1 (AF1) and the DNA binding domain (DBD) of the AR (36) (Fig. 2C). Since AF1 is the predominant transcriptional activation domain of the AR (37-39), this mutant remains constitutively active even in the absence of hormone stimulation (37, 40). When AR∆LBD along with CRKL were co-transfected into AR negative PC3 prostate cells, an enhanced transactivation was observed (Fig. 2C). This was similar to that seen with SRC-1e (also known to interact at the AF1 domain of the AR) (38). Since overexpression of both CRKL and SRC-1e further enhanced activity of AR∆LBD, it may be possible that there is a synergistic effect of both proteins at that region of the AR. The AF1-region also contains several potential phosphorylation sites (41) and interaction domains for co-activators (42-47), therefore, the influence of CRKL could be mediated via two important properties of its SH domains. First, they contain important protein-protein interaction modules; and secondly they are non-catalytic regulators of kinase activity (48). We next assessed if the truncated SH domains of CRKL would still have an effect on the constitutively active AR∆LBD mutant by performing transcriptional reporter assays with the SH2, the N-terminal SH3 (SH3-N) and the C-terminal SH3 (SH3-C) domains of CRKL. We found that all three SH domains were responsible in mediating transcriptional activity of the AR (Fig. 2D). CRKL may therefore potentially assist in the assembly of a heterogeneous multi-protein complex on the AR, involving key kinase signalling factors that have yet to be identified.

Growth factors modulate the CRKL/AR/SRC-1e complex

Studies in the hormone refractory stage of prostate cancer have shown that the AR still remains functionally active in the absence of androgens (49). One of the suggested mechanisms involves a cross-talk between Src/Ras/Erk signal transduction with that of the AR signalling pathway (50-52). Since CRKL is

a known adaptor protein for signal transduction, we investigated if its enhancement of AR activity could be attributed to recruitment of growth factors involved in prostate cancer development (53). We tested the following factors: Platelet Derived Growth Factor (PDGF), Insulin-like Growth Factor-1 (IGF-1), Vascular Endothelial Growth Factor (VEGF), Epidermal Growth Factor (EGF) or the cytokine- Interleukin-6 (IL-6). We transfected PC3 cells either with: (AR + CRKL + SRC-1e); with (AR +SRC-1e) or with (AR alone), and exposed these cells to the different compounds under androgen ablation conditions (1nM androgen + 10μM casodex). We found a significant level of AR transcriptional activity only in cells where CRKL was overexpressed (Fig. 3), suggesting that the AR has the ability to recruit CRKL possibly through an alternative signalling pathway. Consistent with these findings, we also showed that overexpression of CRKL was able to upregulate PSA levels in LNCaP cells in a hormone-free environment or in the presence of casodex (Fig 1J).

To address a possible signalling mechanism influenced by CRKL, we targeted its associated ERK/MAPK signalling pathway with the specific inhibitor U0126 (54). We found that U0126 abrogated phosphorylation of ERK in cells transfected with (AR + CRKL + SRC-1e) (Fig. 4A) and significantly reduced androgen induced AR transcriptional activity in these cells (Fig. 4B). Since numerous growth factors are involved in the activation of the ERK/MAPK pathway, we targeted the EGFR axis by using a specific inhibitor of this receptor (AG1478) (55). We found that targeting EGFR significantly reduced EGF induced AR activity in cells transfected with (AR + CRKL + SRC-1e) (Fig. 4B). Since the biological function of CRKL is regulated by phosphorylation of its SH domains (56); and imatinib is a known inhibitor of this (57, 58) , we subsequently tested the effect of imatinib on androgen induced AR transcription in cells transfected with (AR + CRKL + SRC-1e (Fig. 4B). The inhibitory effect of imatinib and U0126 on androgen mediated AR activity was similar to that seen with AG1478 treatment on EGF mediated AR activity (Fig. 4B).

Since our western blot data shows that phosphorylated CRKL was only affected by imatinib and AG1478 (Fig. 4C); we speculate that, of the numerous

signal transduction pathways that could modulate an alternative AR signalling, CRKL and EGFR could be partly responsible for enhancing this effect. Although EGFR induced AR signalling has already been established (59, 60); we provide data to show that the upregulation of an important factor such as CRKL may be a sufficient driving force to further assist the AR into adopting an alternative signalling pathway.

siRNA knockdown affects AR transcriptional activity and cell proliferation

To gain further insight into the biological and cellular influence of CRKL on AR signalling we performed siRNA knockdown studies in LNCaP cells. After establishing efficient knockdown of CRKL, SRC-1e and AR (Fig. 5A), we used the same siRNA conditions to carry out a reporter assay in response to androgen stimulation (Fig. 5B). Androgen induced AR activity was significantly reduced in LNCaP cells when CRKL, AR and SRC-1e were targeted by siRNA. Since we had already established that endogenously expressed CRKL was recruited with the AR to the enhancer region of PSA (KLK2) in LNCaP cells (Fig. 1F), we next established whether knockdown of CRKL or overexpression of CRKL would have an effect on recruitment of AR to the same enhancer region. A ChIP assay was performed in siRNA-CRKL transfected cells and in cells with transient overexpression of CRKL (pCDNA3.1-CRKL) (Fig. 5C). The amount of ligand activated AR recruited to the enhancer region of PSA (KLK2) was different in cells overexpressing CRKL compared to cells where CRKL expression was knockdown (Fig. 5C).

In summary, we show that the adaptor protein, CRKL, has a dynamic function where in prostate cancer it influences AR signaling via a cooperative interaction with the AR and possibly other co-activators that are yet to be determined. More importantly this interaction appears to be modulated by growth factors in a hormone free environment even in the presence of casodex. There are as yet no published reports that define the role of an adaptor molecule in hormone resistant prostate cancer cells. Bcar1/p130Cas (Breast cancer resistance/p130 Crk- associated substrate) is currently the only Crk associated

protein demonstrated to promote growth and proliferation of anti-estrogen resistant breast cancer cells (61) by involving cell migration, invasion (62, 63) and growth factor receptor signalling (64-67). Our next challenge will be to map the interaction domain of CRKL with the AR, and to establish which other CRKL associated factors are involved along the ERK/MAPK signal transduction pathway to facilitate androgen independent signaling. The possible existence of other coactivators similar to CRKL will have broader implications when further defining the alternative pathways by which the AR adapts in different therapeutic settings.

MATERIALS AND METHODS

Cell Culture

PC3 and LNCaP cells (American Type Culture Collection) were cultured in RPMI-1640 (Sigma) supplemented with 100 units/ml penicillin, 0.1mg/ml streptomycin, 2mmol/L glutamine (Sigma) and 10% fetal bovine serum (Labtech International). PC3wtAR cells (28) were grown in RPMI with 4µg/ml of Geneticin (Gibco). Androgen-free culture conditions were carried out in phenol red free DMEM or RPMI supplemented with charcoal-stripped fetal bovine serum (Labtech International).

Reporter assay

The following plasmids have been described previously: pSG5-SRC-1e (68)**,** pSVAR (ΔLBD) (a.a. 1-653) (36) and pCDNA3.1-CRKL (23). The following were kind gifts to Bevan CL: pSVAR from Brinkmann A (Rotterdam), TAT-GRE-EIB-Luc from Jenster G (Rotterdam). Cells were cultured in 24-well plates for 24 hours followed by transfection using FuGENE6 (Roche Diagnostics), or for LNCaP cells by using Nanofectamin (PAA). The transfected DNA (measured in nanograms per well) included empty pSG5 or empty pCDNA3.1 control plasmids to standardise the amounts of DNA, the reporter TAT-GRE-E1B-Luc (500ng), pdmLacZ-β-Gal (250ng) and the vectors pSVAR (50ng), pSVAR- ΔLBD (50ng), pSG5-SRC-1e (200ng) and pCDNA3.1-CRKL (50-200ng). After incubation for 16 hours, cells were washed and treated with 10nM hormone mibolerone (MB) for 24hours. Cells were washed twice in phosphate buffered saline (PBS) and lysed in reporter lysis buffer (Promega). Extracts were analysed for firefly luciferase activity using the LucLite™ kit (Packard) and values corrected for β-galactosidase activity measured by the GalactoLight Chemiluminescence assay (Tropix).

Inhibition studies

PC3 cells were transfected as described above for 24 hours. For single treatment with growth factors alone: PDGF (20ng), IGF-1 (20ng), VEGF (100ng), EGF (100ng) and IL6 (100ng) were added to the cells for 16 hours prior to harvesting for the reporter assay. Where cells were pre-treated with either MB (10nM) or casodex (Cas) (1μM): compounds were added for 4 hours and washed twice in TBS before adding the growth factors: PDGF (40ng), IGF-1 (40ng), VEGF (200ng), EGF (200ng) and IL6 (200ng) for 4 hours prior to harvesting. Where cells were given a combination of treatment for 16 hours (Cas + MB + growth factors + U0126): 1μM of Cas alone or 1nM of MB and 10μM of Cas combined were added to the cells together with the growth factors at the same concentrations used for the 16 hour incubation period as sated above; and the MAPK/ERK kinase inhibitor U0126 (20μM) prior to harvesting. Where cells were treated with MB (10nM) or the growth factors, and imatinib (5μM), U0126 (20μM) or AG1478(20μM): cells were pre-treated with the growth factors at the same concentrations used for the 4 hour incubation period, as stated above. Cells were washed twice in TBS prior to addition of imatinib, U0126 or AG1478 for a further 4 hours prior to harvesting.

Immunoprecipitation

Immunoprecipitation (IP) was performed as previously described (23) with some modifications. Cells were grown to 80% confluency in a 10 cm culture dish**.** 10nM MB was added for 2 hours before harvesting. Cells were washed twice in ice-cold PBS before incubation for 20 minutes on ice in IP buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% Nonidet P-40, 1mM dithiothreitol and complete protease inhibitor cocktail). The lysates were centrifuged at 14000 rpm for 5 minutes at 4°C. 500μg of total protein extract was then pre-cleared with protein-A/G-Ultralink Resin (Thermo Scientific) for 45 minutes at 4°C prior to incubation with rabbit primary antibody against AR or CRKL (Santa Cruz) overnight at 4°C. The immune complex was then precipitated with protein-A/G-Ultralink resin for 1 hour

at 4°C, washed three times in a high salt wash buffer (20mM Tris pH 8, 150mM NaCl, 1mM EDTA, 0.1% NP40) and resuspended in laemmli SDS loading buffer for separation by SDS-PAGE.

Immunoprecipitating CRKL from Cytoplasmic and nuclear cell extracts

Cells (at a density of $0.7x10^6$ cells/ml) were grown for 16 hours in $10cm^2$ dish and transfected with pCDNA3.1-CRKL or empty vector control for 24 hours followed by hormone treatment for a further 2 hours. Cells were washed twice in cold TBS and then gently scraped into an eppendorf. Cells were then resuspended in 100µl of cold homogenisation buffer (10mM Hepes, pH7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM DTT and 0.5mM PMSF). Harvested cells were allowed to swell on ice for 15 minutes before being lysed by the addition of 10µl of a 10% solution of NP-40 followed by 10seconds of vigorous vortexing. The resulting nuclear pellet was then centrifuged at 13,000rpm for 30 seconds with the cytoplasmic supernatant removed. The nuclear pellet was then washed three times in homogenisation buffer containing NP40 and resuspended in 100µl of the same buffer. The nuclear proteins were solubilised by sonication. 100µl of the cytoplasmic and nuclear extracts were resuspended in 400 µl of IP buffer (50mM Tris-HCl pH 8.0, 150mM NaCl, 1% Nonidet P-40, 1mM dithiothreitol and complete protease inhibitor cocktail). After a pre-clearing stage with protein-A/G-Ultralink Resin (Thermo Scientific) for 45 minutes, 1µg of anti-CRKL (Santa Cruz) or IgG as negative control was added to each sample and incubated overnight at 4˚C. The immune complex was then precipitated with protein-A/G-Ultralink Resin for 1 hour at 4˚C, washed three times in PBS and resuspended in laemmli SDS loading buffer for separation by SDS-PAGE.

Immunofluorescence

PC3wtAR cells were grown to 50% confluency in normal RMPI media on sterile glass coverslips in 24-well plates. For transient overexpression of CRKL, 200ng of pCDNA3.1-CRKL was transfected using FuGENE6 (Roche) according to the manufacturer's instructions. Cells were then grown for an additional 24 hours.

The coverslips were then washed in PBS three times, fixed in 4% paraformaldehyde for 20 minutes and solubilised in 0.2% TritonX100 for 20 minutes. The coverslips were washed three times followed by treating with 10% foetal calf serum for 45 minutes. Rabbit anti-AR (1:200) (Santa Cruz); and Mouse anti-CRKL (1:50) (Cell Signalling Technology) were added to the cells in 10% FCS for one hour. Cells were washed three times in PBS and 10% FCS added for 15 minutes before incubation with Alexa-488 conjugated goat anti-mouse (1:600) or Alexa-594 conjugated chicken anti-rabbit secondary antibody(1:600) (Molecular Probes) for one hour. After five washes in PBS, coverslips were mounted on glass slides with Vectashield containing 4'6'-diamidino-2-phenylindole (DAPI) (Vector labs). Slides were visualised on a Leica DM4000 at 100x magnification. An average of 10 images per treatment was captured.

Immunohistochemistry

Prostate samples from 6 separate patients were obtained from AccuMAx Array (Cepheid). The formalin fixed, paraffin embedded prostate tissue were dewaxed in xylene and rehydrated in decreasing concentrations of ethanol. Endogenous peroxidise activity was blocked using 2% hydrogen peroxide. Antigen retrieval was carried out by microwaving at 750W in 0.01M trisodium citrate, pH6, for 5 minutes. Sections were blocked with PowerBlock (BioGenex) (1:10 dilution) for 3 hours at room temperature and incubated with the primary antibody: anti-human CRKL (Santa Cruz) (1:250) overnight at 4˚C. After several washing, sections were incubated with biotinylated anti-rabbit IgG (DAKO) (1:200 dilution) for 45 minutes, followed by peroxidise conjugated with streptavidin (DAKO) (1:100 dilution) for 30 minutes. Sections were then washed and enzyme activity was developed in 1mg of 3,3'diaminobenzidine tetrahydrochloride (DAKO) per ml and counterstained with hematoxylin (Vector Laboratories). All images were processed at x40 magnification with an Olympus CKX41 microscope mounted with a CC12 Olympus UCMAD3 camera.

Chromatin immunoprecipitation (ChIP)

LNCaP cells were seeded at a ratio of $7x10^5$ cells/ml in serum starved media for 24 hours in 6 well plates followed by transient overexpression using Lipofectamine RNAiMAX (Invitrogen) with (200ng pCDNA3.1-CRKL), siRNA knockdown of CRKL (Dharmacon) as previously mentioned or un-transfected for 48 hours. After a total of 72 hours, cells were treated with 100nM MB and 200ng EGF for 2 hours before cross-linking with formaldehyde (Sigma) for 10 minutes at 37˚C. ChIP was performed using the Upstate Chromatin Immunoprecipitation kit (Upstate) following the manufacturer's instructions. Briefly, cross-linked cells were lysed and sonicated for chromatin fragments of about 1000bp. Anti-AR (Santa Cruz), anti-CRKL (Cell signalling) or Rabbit-IgG (Jackson Laboratories) were incubated with the fragments to precipitate out their corresponding peptides cross-linked with the DNA fragments. The cross-linked DNA was then recovered by protease treatment and phenol-chloroform extraction and semi-quantitative PCR performed using primers designed to anneal either side of an ARE in the *KLK2* enhancer region (For- 5' TTGAAAGCAGACCTACTCTGGA-3'; Rev-5'CTGGACCATCTTTTCAAGCAT-3') (33).

RT PCR

LNCaP cells were seeded at a density of $0.7x10^6$ cells/ml in 24 well plates with charcoal stripped FCS/ RPMI media for 16 hours before transfection with either 200ng of pCDNA3.1-CRKL or the control vector (empty pCDNA3.1) as a negative control. 24 hours later, cells were either treated with 10nM of mibolerone (MB), Casodex (1µM) or its carrier ethanol for a further 24 hours before harvesting. RNA was recovered using the RNAqueous-Micro kit (Ambion) following the manufacturer's recommendation. The RNA was quantified using a Nanodrop 2000 micro-sample quantitator. 1µg of total RNA from each sample was reverse transcribed using the One Step RT-PCR kit from Qiagen following the manufacturer's recommendation. Expression of human PSA was measured semi-quantitatively by PCR using primer pairs: Forward- 5'-TTGTCTTCCTCACCCTGTCC-3' and Reverse-5'TCACGCTTTTGTTCCTGATG-

3’ for 25 cycles at 94˚C for 1min, 58˚C for 45 seconds and 72˚C for 1min. GAPDH was used as a loading control and amplified by PCR using the primer pairs: Forward- 5’-GTGAAGGTCGGAGTCAACG-3’ and Reverse-5’-GGTGAAGACGCCAGTGGACTC-3’ for 30 cycles at 94˚C for 45 sec, 60˚C for 45 seconds and 72˚C for 1min.

siRNA targeted knockdown

LNCaP cells were seeded in androgen depleted media at a density of $7x10^5$ cells/ml in a 24 well plate for 24 hours and transfected with 40nM final concentration of siRNA AR (Dharmacon) or 200nM final concentration of siRNA CRKL (Dharmacon), SRC-1e (Dharmacon) and scrambled siRNA (Ambion) using Nanofectamin (PAA). Cells were incubated for 72 h with siRNA for efficient knockdown. For reporter assay data, cells were transfected with the relevant siRNA together with TAT-GRE-EIB-Luc and pdmLacZ-β-Gal for 48 hours before addition of ligand for a further 24 hours before harvesting.

Western blotting

All cell extracts were prepared at a concentration of 30μg per well in SDS-PAGE loading buffer and loaded on to Novex 4-20% Tris-Glycine Gels (Invitrogen). Under denaturing conditions, proteins were separated by gel electrophoresis and transferred onto nitrocellulose membrane using a semi-dry blotting apparatus (Trans-Blot SD Semi-Dry, Bio-Rad). The membranes were blocked in TBS containing 5% non-fat milk for 1 hour before incubating with primary antibodies for 1 hour at room temperature. Antibodies used were mouse anti-CRKL (Cell Signalling), rabbit anti CRKL (Santa Cruz), rabbit anti- AR (N20) (Santa Cruz), rabbit anti SRC-1e (Cell Signalling), rabbit anti phospho CRKL (Tyr 207) (Cell Signalling), rabbit anti ERK (Cell Signalling) and rabbit anti Phospho ERK (Cell Signalling). After subsequent membrane washing, detection was carried out using the horseradish peroxidase conjugated anti-mouse, anti-rabbit, or mouse IgG secondary antibody (1:5000) (Jackson Immuno Research) and incubated for

1 hour at room temperature. Following further washes, proteins were visualised using the enhanced chemiluminescence detection system (Amersham).

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Figure legend

FIGURE. 1. CRKL and AR complex. **A.** Co-immunoprecipitation of AR and CRKL. Cell lysates were immunoprecipitated with anti-AR and anti-CRKL antibodies and probed with their respective antibodies for a western blot analysis. PC3wtAR (stable cell line expressing AR), PC3 (androgen receptor negative), and **B.** LNCaP (endogenously expressing the AR) were used. Representative data are shown. (The slight band shift seen from LNCaP cells (B) is attributed to the high salt buffer used for the sepharose bead washing conditions following immunoprecipitation). **C.** Subcellular localisation of CRKL in PC3wtAR cells was assessed by isolating the cytoplasmic and nuclear compartments followed by immunoprecipitating and probing with anti-CRKL antibody for a western blot analysis. **D.** Confocal microscopy of PC3wtAR cells to determine localisation of CRKL and AR endogenously (control) or with CRKL overexpressed (Transfection with pCDNA3.1-CRKL). **E.** Immunohistochemistry of prostate tissue biopsies from patients with advanced prostate cancer (Gleason score of 9 at stages T3A and T3B) viewed at x40 magnification. CRKL staining appears more dense and nuclear relative to corresponding matched normal tissue. **F.** Chromatin immunoprecipitation from LNCaP cell extracts performed with anti-AR and anti-CRKL antibodies at an endogenous androgen-responsive element in the enhancer region of Kallikrein-2. **G.** Western blot analysis of PC3wtAR cell extract to show upregulation of CRKL following transfection of pCDNA3.1-CRKL relative to its control vector. **H.** AR driven luciferase reporter assay with exogenously expressed AR and CRKL in COS cells and, **I.** PC3 cells. Results are the mean ± SEM (from 4 independent duplicate experiments) and statistically significant, $p < 0.05$ (Two tailed Student's t test). **J.** Total RNA from LNCaP cells overexpressing CRKL and treated with mibolerone (MB) or casodex (cas) were reverse transcribed for PCR amplification of PSA and GAPDH for semi quantitative analysis. Results are representative of three individual experiments.

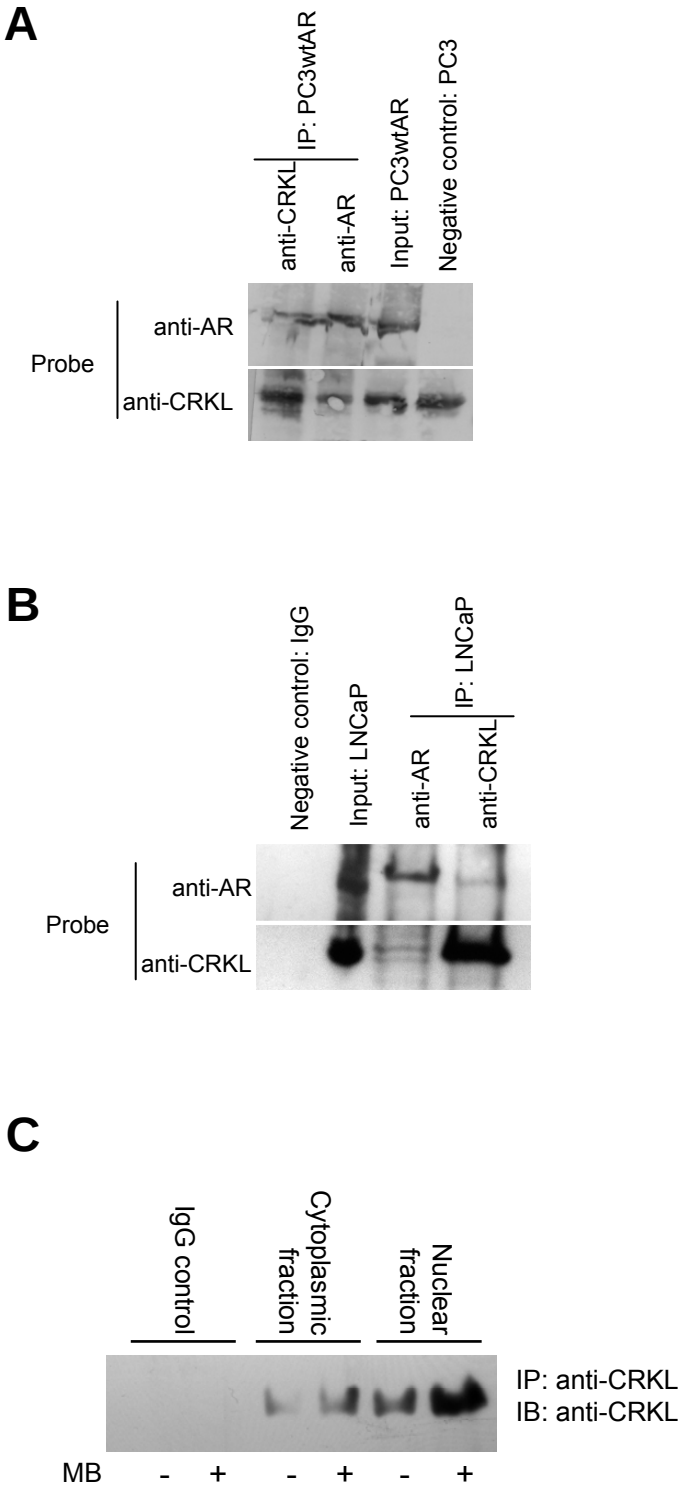
FIGURE. 2. Transactivation of CRKL, AR, and SRC-1e complex. **A.** Immunoprecipitation of CRKL, AR, and SRC-1e complex. LNCaP cell lysates were immunoprecipitated with anti-AR antibody or with IgG as control and probed with anti-AR, anti-CRKL, and anti-SRC-1e antibodies for a western blot analysis. Representative data is shown. (The slight band shift seen in the IP lane is attributed to the high salt buffer used for the sepharose bead washing conditions following immunoprecipitation). **B.** Androgen stimulated AR luciferase reporter assay where AR, CRKL, and SRC-1e are co-transfected into LNCaP and PC3 cells. **C.** Enhanced transactivation of the AR∆LBD mutant following overexpression of CRKL alone or CRKL with SRC-1e, or **D.**, following overexpresion of the CRKL fragments: SH2, SH3(N) and SH3(C). The AR∆LBD mutant is a constitutively active and nuclear AR mutant with the LBD and AF2 domains (36). Results are the mean ± SEM from 3 independent duplicate experiments. The schematic diagrams show the AR∆LBD mutant and the location of CRKL SH domains.

FIGURE. 3. Activation of the CRKL/AR/SRC-1e complex by growth factors by-passes casodex inhibition. AR driven luciferase reporter assay in transfected PC3 cells transiently transfected either with (AR + CRKL + SRC-1e) or (AR + SRC-1e) or (AR alone). Growth factors: PDGF (40ng), IGF-1(40ng), VEGF (200ng), EGF(200ng) and the cytokine, IL6(200ng) induced AR activity was measured in the presence of casodex (Cas) (10µM) and low level mibolerone (MB) (1nM). Increased AR activity was observed only in cells where CRKL was overexpressed. Results are the mean ± SEM from 3 independent duplicate experiments.

FIGURE. 4. The CRKL/AR/SRC-1e complex associated with the ERK/MAPK signalling**. A.** Western blot analysis of transfected PC3 cells after treatment with the androgen MB (10nM), imatinib (5μM) (Inhibitor of phosphorylated CRKL), UO126 (20μM) (ERK/MAPK inhibitor) and AG1478 (20μM) (EGFR inhibitor) to show its effect on the phosphorylation status of ERK. **B.** AR driven luciferase reporter assay in PC3 cells transiently expressing CRKL/AR/SRC-1e complex and treated with mibolerone or the growth factors EGF in the presence of the inhibitors for CRKL (imatinib) and ERK/MAPK (U0126) and EGF (AG1478). Results are the mean ± SEM from 3 independent duplicate experiments. **C.** Western blot analysis of transfected PC3 cells after treatment as mentioned in (A) to show its effect on the phosphorylation status of CRKL.

FIGURE. 5. siRNA knockdowns of endogenous AR, CRKL and SRC-1e affects AR activity**. A.** Western blot for targeted knockdown of CRKL, SRC-1e and AR in LNCaP cells 72 hours post transfection. β-actin is used as a loading control. **B.** AR driven luciferase reporter assay of ligand activated AR following targeted knock down of either CRKL, AR, SRC-1e or knock down of all three (CRKL/ AR/SRC-1e) in LNCaP cells. **C.** ChIP analysis of the PSA (*KLK2)* enhancer region in mibolerone (MB) treated LNCaP cells following control (scramble), overexpression of CRKL (pCDNA-CRKL) or targeted knockdown of CRKL by siRNA. Representative data is shown. Results for the reporter assay are the mean ± SEM from 3 independent duplicate experiments.

FIGURE 1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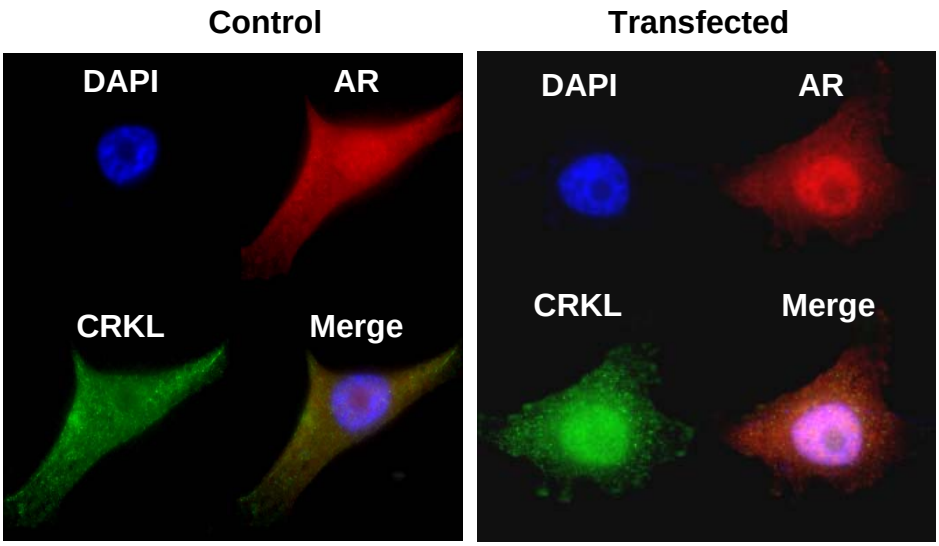
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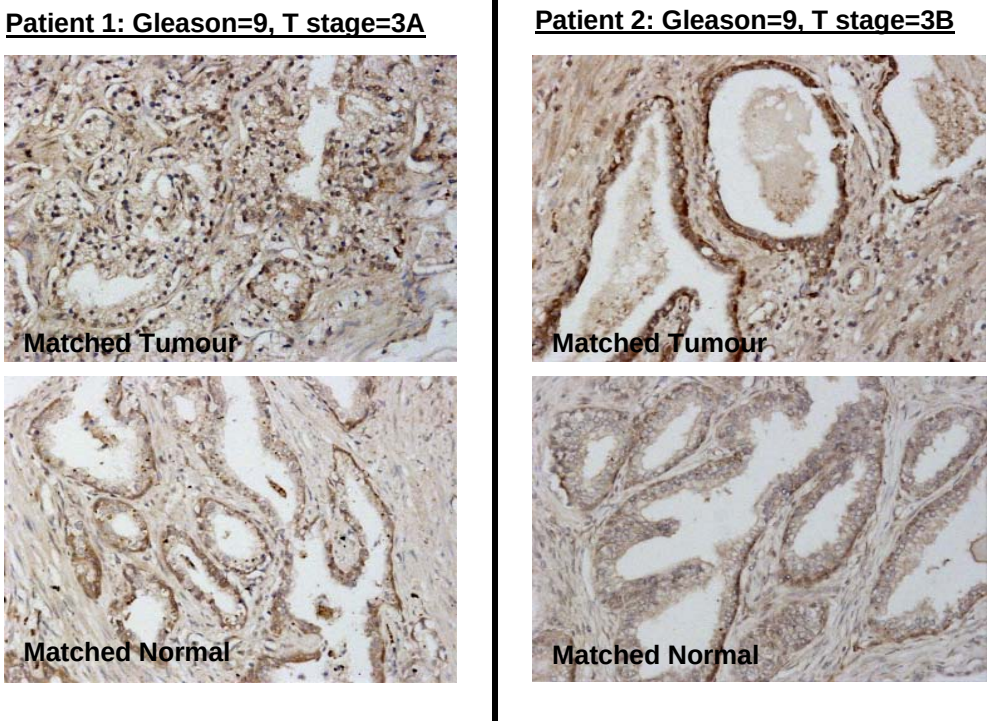
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Matched Normal
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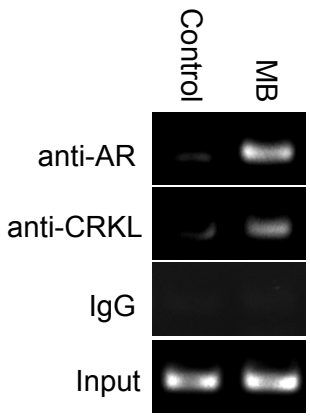
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Input

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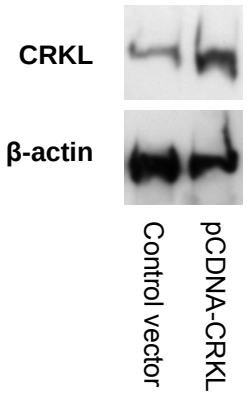
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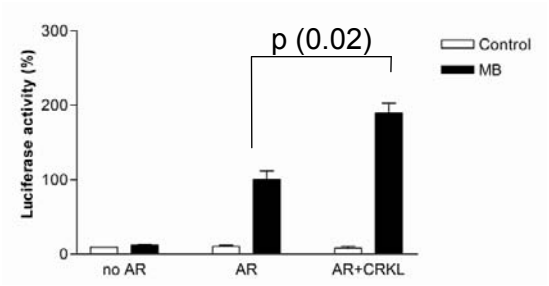
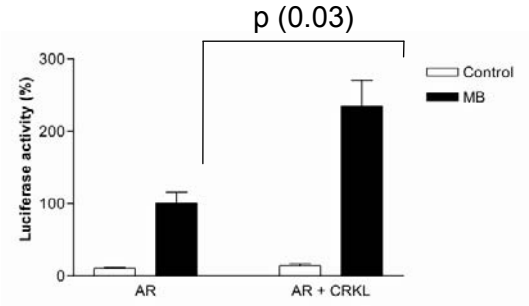

p (0.02)
Control
MB
Luciferase activity (%)
300
200
100
0
no AR
AR
AR+CRKL

I


p (0.03)
Control
MB
Luciferase activity (%)
300
200
100
0
AR
AR + CRKL

J

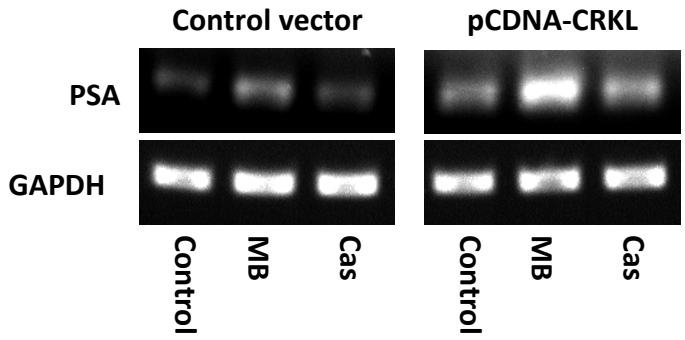

Control vector
pCDNA-CRKL
PSA
GAPDH
Control
MB
Cas
Control
MB
Cas

FIGURE 2

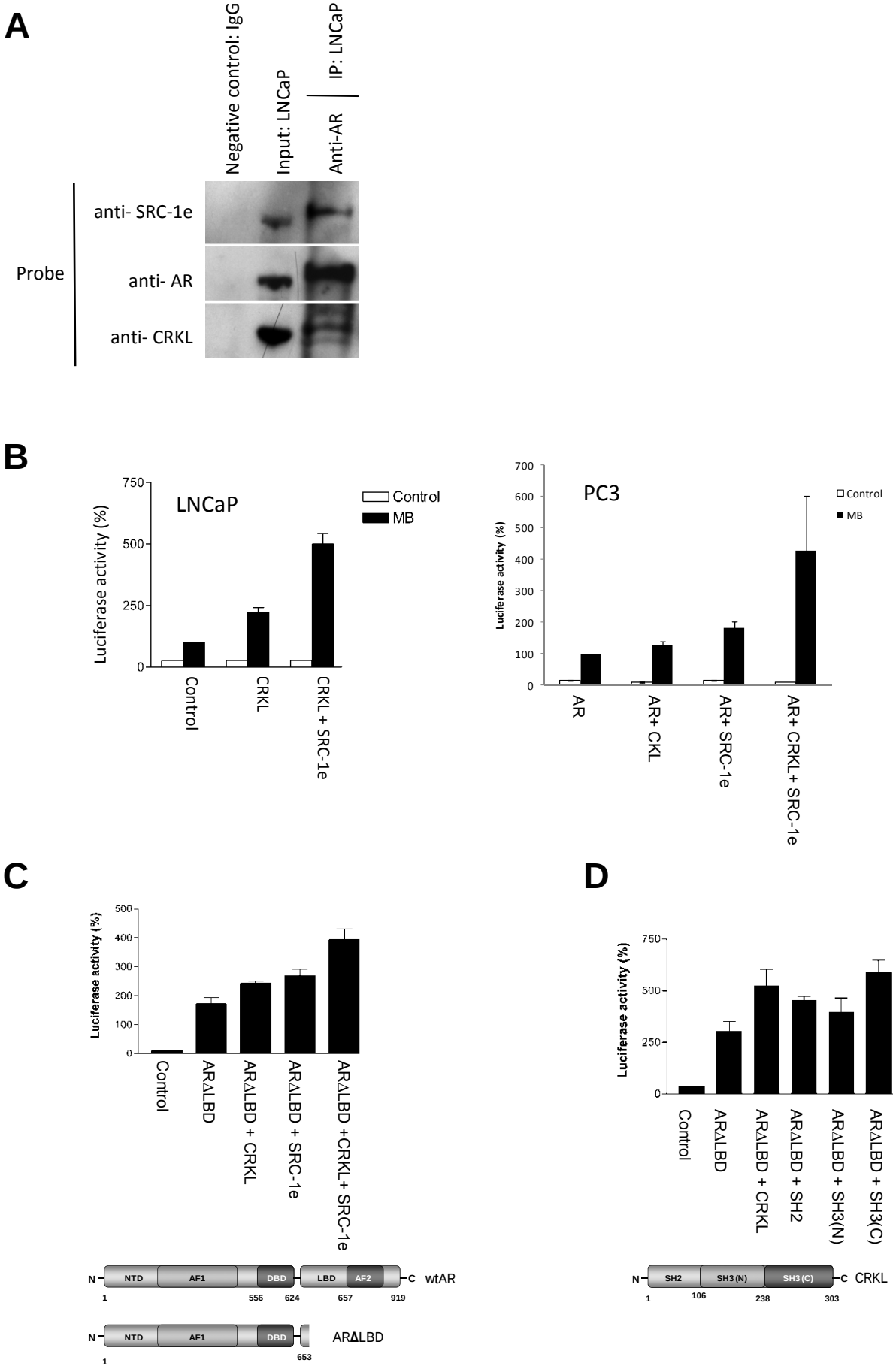

A
Negative control: IgG
Input: LNCaP
IP: LNCaP
Anti-AR
Probe
anti- SRC-1e
anti- AR
anti- CRKL
B
LNCaP
Control
MB
Luciferase activity (%)
750
500
250
0
Control
CRKL
CRKL + SRC-1e
PC3
700
600
500
400
300
200
100
0
AR
AR+ CKL
AR+ SRC-1e
AR+ CRKL+ SRC-1e
C
500
400
300
200
100
0
Control
ARΔLBD
ARΔLBD + CRKL
ARΔLBD + SRC-1e
ARΔLBD +CRKL+ SRC-1e
N
NTD
AF1
DBD
LBD
AF2
C
wtAR
1
556
624
657
919
ARΔLBD
653
D
750
500
250
0
Control
ARΔLBD
ARΔLBD + CRKL
ARΔLBD + SH2
ARΔLBD + SH3(N)
ARΔLBD + SH3(C)
SH2
SH3 (N)
SH3 (C)
CRKL
106
238
303

FIGURE 3

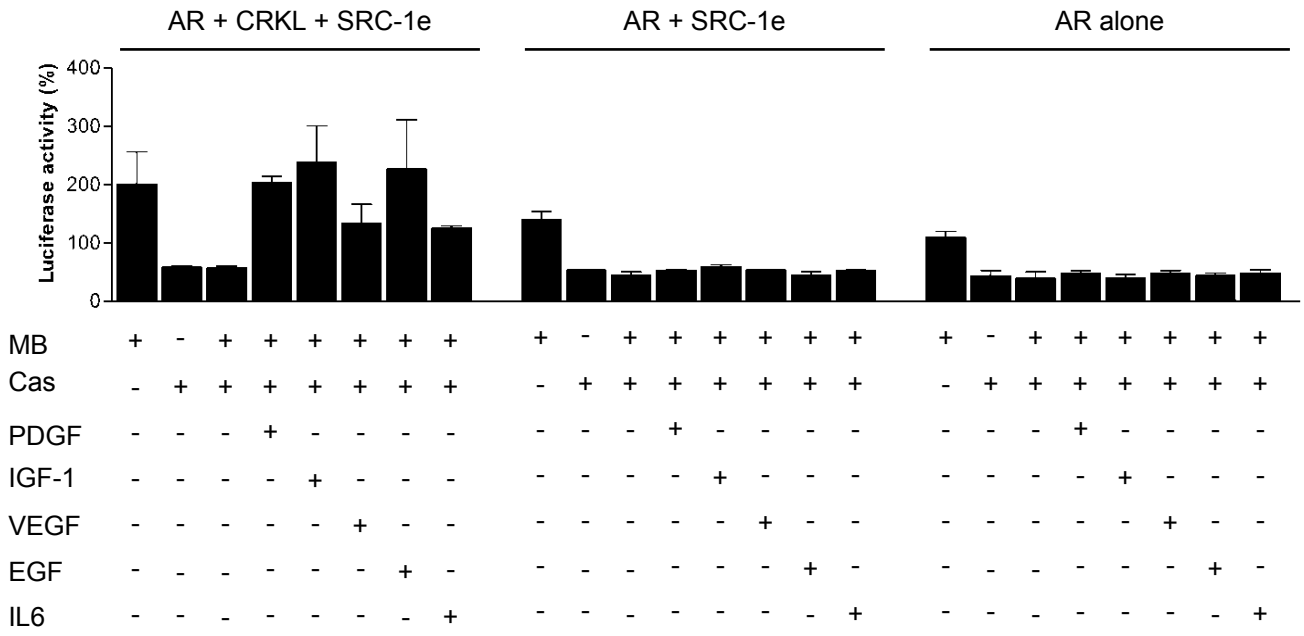
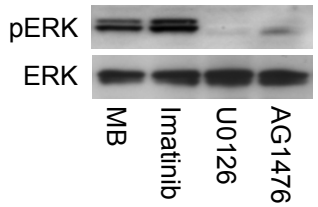
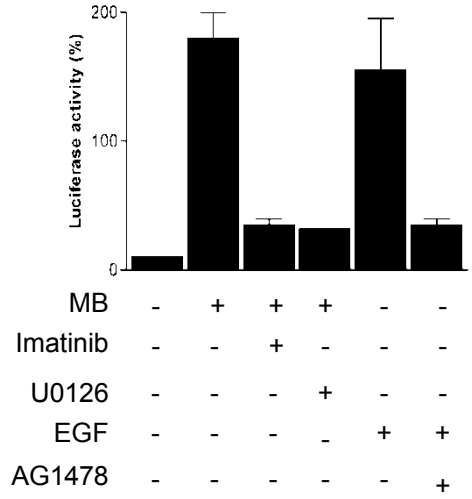

AR + CRKL + SRC-1e
AR + SRC-1e
AR alone
Luciferase activity (%)
400
300
200
100
0
MB + - + + + + + + + - + + + + + + + - + + + + + + +
Cas - + + + + + + + - + + + + + + + - + + + + + + +
PDGF - - - + - - - - - - - + - - - - - - - + - - - -
IGF-1 - - - - + - - - - - - - + - - - - - - - + - - -
VEGF - - - - - + - - - - - - - + - - - - - - - + - -
EGF - - - - - - + - - - - - - - + - - - - - - - + -
IL6 - - - - - - - + - - - - - - - + - - - - - - - +

Figure 4

A


pERK
ERK
MB
Imatinib
U0126
AG1476

B


Luciferase activity (%)
200
100
0
MB - + + + - -
Imatinib - - + - - -
U0126 - - - + - -
EGF - - - - + +
AG1478 - - - - - +

C

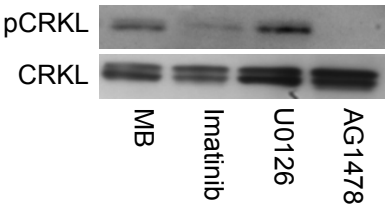
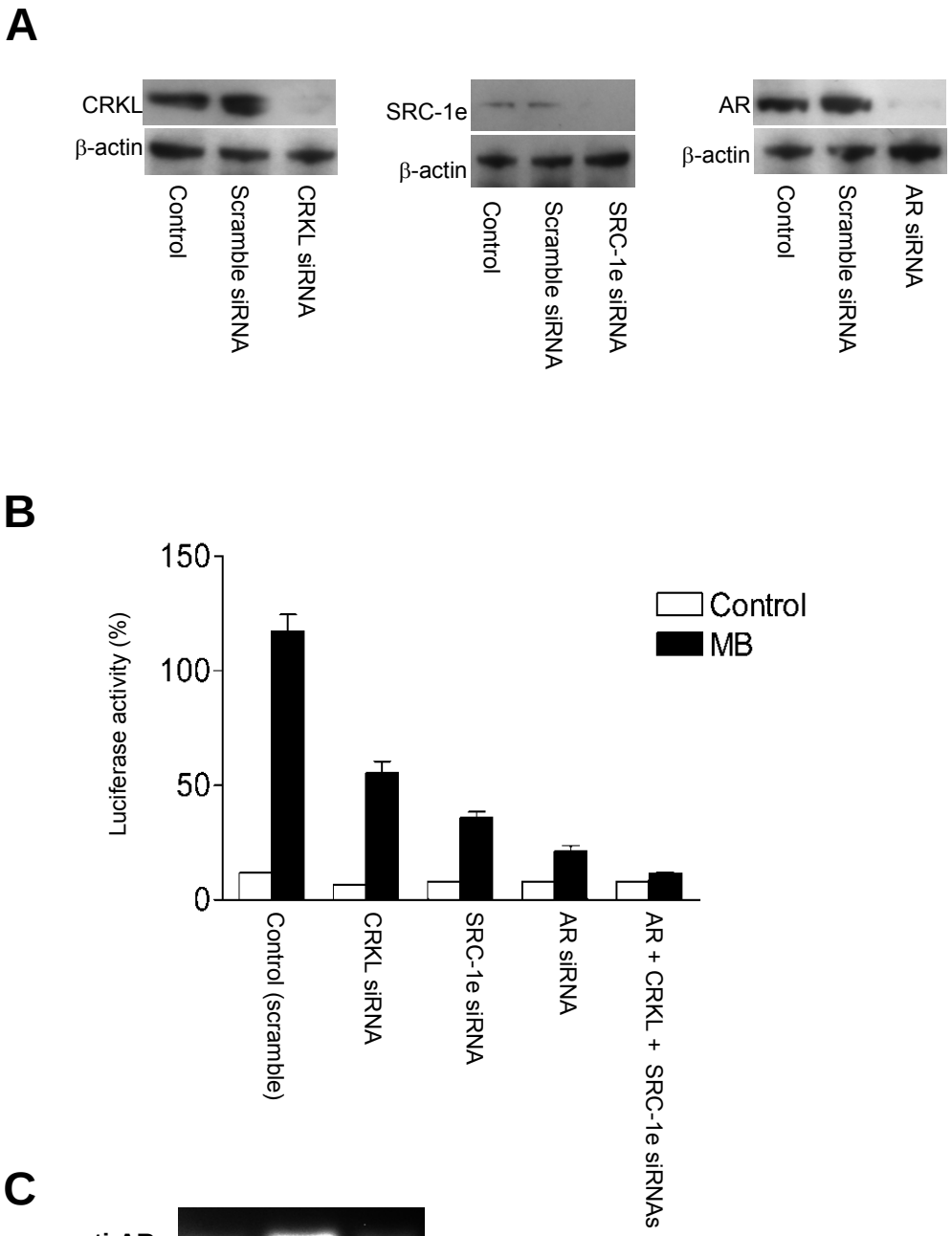

pCRKL
CRKL
MB
Imatinib
U0126
AG1478

Figure 5


A
CRKL
β-actin
Control
Scramble siRNA
CRKL siRNA
SRC-1e
β-actin
Control
Scramble siRNA
SRC-1e siRNA
AR
β-actin
Control
Scramble siRNA
AR siRNA
B
Luciferase activity (%)
150
100
50
0
Control
MB
Control (scramble)
CRKL siRNA
SRC-1e siRNA
AR siRNA
AR + CRKL + SRC-1e siRNAs
C
anti-AR
anti-CRKL
IgG
Input
Control (scramble)
pCDNA-CRKL
CRKL siRNA